MEDICAL UNIVERSITY OF VIENNA

The role of Siglec-G in atherosclerosis and liver inflammation

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"Doctor of Philosophy – PhD"

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DECLARATION

The doctoral candidate, Sabrina Gruber, conducted her PhD studies under the supervision of Univ. Prof. Christoph J. Binder, MD, PhD at the Department of Laboratory Medicine (KILM) and the Research Center for Molecular Medicine (CeM-M-) of the Austrian Academy of Sciences.

Most of the experiments were performed in the laboratory of Christoph Binder at the above mentioned institutes. The thesis was written in full by the author and the work presented in the following publications was largely done by the author.

The publication arising from this thesis is entitled “Sialic acid binding immunoglobulin-like lectin G promotes atherosclerosis and liver inflammation by suppressing the protective functions of B-1 cells“ and the contributions of co-authors for the manuscript are as following: Sabrina Gruber designed and performed experiments, analyzed and interpreted data, and wrote the manuscript. Tim Hendrikx assisted with experimental work and data analysis. Dimitris Tsiantoulas assisted with experimental work and provided intellectual input. Maria Ozsvar-Kozma and Laura Göderle assisted with technical help. All other co-authors, Lars Nitschke, Joseph L. Witztum, Ziad Mallat and Ronit Shiri-Sverdlov provided material support or critically revised the manuscript.

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TABLE OF CONTENTS

LIST OF FIGURES

Introduction

Published Figures

LIST OF TABLES

ABSTRACT – English

Atherosclerosis is recognized as a chronic inflammatory disease of the vascular wall and is initiated and sustained by hypercholesterolemia. This consequently leads to the generation of oxidized LDL (OxLDL) as well as other metabolic by-products that are capable of triggering inflammation. It is known that specific immune responses can modulate the inflammatory response during atherogenesis. The inhibitory receptor sialic acid-binding immunoglobulin-like lectin G (Siglec-G) acts as a negative regulator of the functions of several immune cells, such as myeloid cells and B-1 cells. In my thesis I identified that deficiency of Siglec-G in cholesterol-fed *Ldlr*$^{-/-}$ mice inhibits atherosclerotic plaque formation and diet-induced liver inflammation. Moreover, I demonstrate that selective deficiency of Siglec-G specifically in B cells alone confers these protective effects. B-1 cell-derived natural IgM antibodies with specificity for OxLDL were significantly elevated in the plasma and peritoneal cavity of Siglec-G-deficient mice. In line with the neutralizing capacity of OxLDL-specific IgM antibodies, I have demonstrated that Siglec-G-deficient mice are protected from OxLDL-induced sterile inflammation. Taken together, Siglec-G promotes atherosclerosis and liver inflammation by suppressing the protective anti-inflammatory effector functions of B cells.

ABSTRACT – Deutsch

Es gilt heute als gesichert, dass Hypercholesterinämie eine wichtige Rolle bei der Entstehung von Atherosklerose spielt. Dies führt im Weiteren zur Entstehung von oxidierten LDL (OxLDL) als auch anderen metabolischen Abbauprodukten, welche wiederum Entzündungen hervorrufen können. Spezifische Immunmechanismen spielen eine wesentliche modulierende Rolle bei der Entzündungsantwort während der Atherogenese. Der inhibitorische Rezeptor *s*ialic acid-binding immunoglobulin-like lectin G (Siglec-G) stellt ein Negativregulator von verschiedenen Immunzellen dar, welche Zellen der myeloischen Reihe und B-1 Zellen umfasst. Im Rahmen dieser Arbeit konnte ich zeigen dass das Fehlen von Siglec-G in einem Mausmodell der Atherosklerose zur Verminderung von atheroskerotischen Plaques als auch zu verringerter Steatose-induzierter Leberentzündung führt. Es konnte weiters nachgewiesen werden, dass die selektive Deletion von Siglec-G in B Zellen alleine ausreichend ist um diese protektiven Wirkungen herbeizuführen. Weiters waren die Titer jener natürlichen Antikörper, die spezifisch gegen OxLDL gerichtet sind und welche von B-1 Zellen produziert werden, sowohl im Plasma als auch in der Peritonealhöhle von Siglec-G-defizienten Mäusen signifikant erhöht. In Übereinstimmung mit einer neutralisierenden Funktion von OxLDL-spezifischen IgM Antikörpern waren Siglec-G-defiziente Mäuse von OxLDL-induzierter steriler Entzündung geschützt. Demzufolge fördert Siglec-G die Entstehung von Atherosklerose und Leberentzündung indem es die schützenden anti-inflammatorischen Effektorfunktionen von B Zellen supprimiert.

PUBLICATION ARISING FROM THIS THESIS

Sialic acid-binding immunoglobulin-like lectin G promotes atherosclerosis and liver inflammation by suppressing the protective functions of B-1 cells.

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ABBREVIATIONS

4-HNE	4-hydroxynonenal
AAP	acetaminophen
ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
AGE	advanced glycation-end
AP	alkaline phosphatase
apoB100	apolipoprotein B100
ApoE	apolipoprotein E-deficient
ASC	apoptosis-associated speck-like protein containing CARD
BAFF	B cell activating factor
BCR	B cell receptor
BMT	bone marrow transplantation
Breg	B regulatory cells
CAD	carotid artery disease
Ccl5	C-C motif ligand 5
CDKN2B	cyclin-dependent kinase inhibitor 2B
CLP	cecal ligation and puncture
CRP	C-reactive protein
CuOx-LDL	copper-oxidized LDL
CVD	cardiovascular disease
Cxcl1	C-X-C motif ligand 1

Cxcl2	C-X-C motif ligand 2
DAMPs	damage-associated molecular patterns
DCs	dendritic cells
FH	familial hypercholesterolemia
FO	follicular
GWAS	genome-wide association studies
i.p.	intraperitoneal
Icam	intercellular adhesion molecule
Il	interleukin
IRA	innate response activator
ITIM	immunoreceptor tyrosine-based inhibition motif
LDL	low-density lipoprotein
LDLR	LDL receptor
LPS	lipopolysaccharide
MDA	malondialdehyde
MertK	mer receptor tyrosine kinase
MFGE8	milk fat globulin E8
MI	myocardial infarction
MPO	myeloperoxidase
MZB	marginal zone B cells
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis

OSE	oxidation-specific epitopes
OxLDL	oxidized LDL
OxPAPC	oxidized phospholipid oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine
PAI-1	plasminogen activator inhibitor-1
PAMPs	pathogen-associated molecular patterns
pDC	plasmacytoid dendritic cells
PEC	peritoneal exudates cells
PLF	peritoneal lavage fluid
POVPC	1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine
PRRs	pattern recognition receptors
PTX3	pentraxin 3
RA	rheumatoid arthritis
RLU	relative light units
SAA	serum amyloid A
SHP-1	src homology 2 domain containing protein tyrosine phosphatase 1
Siglec-G	sialic acid-binding immunoglobulin-like lectin G
SLE	systemic lupus erythematosus
SRA	scavenger receptor A
SRs	scavenger receptors
TCRs	T cell receptors
TG2	transglutaminase-2
TLR	toll-like receptor

Tnf-α	tumor necrosis factor-alpha
TRAIL	TNF-related apoptosis-inducing ligand
Tregs	regulatory T cells
Vcam	vascular cell adhesion protein
β2GP1	beta2glycoprotein1

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1. INTRODUCTION

1.1. Atherosclerosis

1.1.1. The pathomechanism of atherogenesis

Cardiovascular disease (CVD) is the leading cause of death worldwide[1, 2]. The main underlying pathology of CVD is atherosclerosis, which is a chronic inflammatory disease of the artery wall that leads to formation of plaques. Rupture of such plaques causes atherothrombotic events such as heart attacks and strokes. Several non-modifiable and modifiable risk factors for atherosclerotic CVD have been identified, including family history, ethnicity, and age as well as smoking, hypertension, obesity, type II diabetes, and hypercholesterolemia[3, 4]. High LDL cholesterol levels and low HDL cholesterol are key risk factors in the pathogenesis of atherosclerotic plaques. High plasma LDL can be a result of an autosomal dominant disorder, called familial hypercholesterolemia (FH), which is caused by genetic mutations in the LDL receptor (LDLR) or apolipoprotein B100 (apoB100) [5]. In addition, gain of function mutations in the enzyme proprotein convertase subtilisin/kexin type 9 (PCSK9), which degrades LDL receptors, can also result in hypercholesterolemia [5]. However, in a majority of cases high LDL cholesterol levels are a result of a combination of polygenic and life-style factors. Of note, genome-wide association studies (GWAS) do not support a causal role for HDL cholesterol levels in cardiovascular disease [6].

When LDL enters the artery wall, it becomes trapped in the subendothelial space, where it undergoes several types of modifications including sequential steps of oxidation[7-9]. The accumulation of oxidized LDL (OxLDL) in the intima and the associated activation of arterial endothelial cells represent initiating steps of atherogenesis. Infiltrating macrophages take up modified LDL particles via scavenger receptors, leading to the generation of lipid-laden foam cells that are hall-mark cells of atherosclerotic lesions of all stages[10]. In the initial stages of plaque formation lesions appear as so-called “fatty streaks”, which can then further progress into more complex plaques. Lesions of all stages display signs of inflammatory activation and are characterized by the recruitment of T lymphocytes secreting several pro-inflammatory cytokines (e.g. IFN-γ).

HDL on the other side, exerts a variety of other anti-atherogenic properties such as the inhibition of lipid oxidation, restoration of endothelial function as well as other anti-inflammatory effects[6]. Indeed, HDL has been shown to dampen OxLDL-induced expression of cytokines and to modulate the recruitment and adhesion of monocytes. In addition, HDL has been shown to inhibit antigen presentation as well as lymphocyte proliferation. Thus, HDL seems to have effects on both innate and adaptive immune responses, which needs to be elucidated in future studies [11, 12]. Moreover, it is critically involved in controlling the cellular cholesterol levels by promoting the reverse cholesterol transport from peripheral tissues, such as the vasculature, to the liver. The ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1) on macrophages mediate the efflux of cellular cholesterol onto HDL and its major protein ApoA-I[13]. However, if HDL becomes dysfunctional due to chemical and structural changes, it can also become pro-inflammatory, and even propagate oxidation of LDL, enhance vascular inflammation, and impair cholesterol efflux[6]. Indeed, at a certain stage macrophage foam cells become unable to maintain cellular cholesterol homeostasis by mediating HDL-dependent cholesterol-efflux, and undergo apoptosis and/or necrosis, which consequently results in the development of an acellular necrotic core inside the lesion[7, 14, 15]. These advanced lesions are further marked by proliferation of smooth muscle cells that promote plaque stabilization by the production of extracellular matrix and the secretion of collagen to form a fibrous cap covering acellular necrotic areas. In part triggered by inflammatory stimuli and the secretion of matrix metalloproteinases, fibrous caps become increasingly thin and prone to rupture, ultimately resulting in the release of pro-coagulant lipids and tissue factor into the circulation that trigger thrombosis. This results in typical adverse clinical effects, such as myocardial infarction (MI) and stroke[16]. Interestingly, recent studies have indicated that an increasing number of cases of myocardial infarction are nowadays caused by plaque erosion rather than plaque rupture[17]. Plaque erosion occurs in the absence of plaque rupture and arises in lesions without a large lipid core and few inflammatory cell infiltrates[18].

1.1.2. Atherosclerosis and inflammation

It is now evident that inflammation plays a pivotal role in the development of atherosclerosis and that inflammation and disturbed lipid metabolism both feed into this chronic inflammatory process in the vascular wall[16, 19-21]. This specific interplay is thought to mediate both the persistence of initiating triggers as well as an impaired resolution of inflammatory responses.

Moreover, a variety of innate and adaptive immune functions have been identified as modulating factors that influence the inflammatory process via several cellular as well as non-cellular players[9, 22].

The prominent role of inflammation in the initiation and progression of atherosclerotic lesions as well as in - the clinically most relevant - plaque rupture is well established[23-25]. Besides clear histopathological evidence showing infiltration of inflammatory cells, which are activated and produce cytokines, many epidemiological studies, as well as data from GWAS support a causal role for inflammation in atherosclerosis. For example, more than 20 prospective cohort studies have revealed clinical evidence for high sensitive measurements of the acute phase reactant C-reactive protein (CRP), which is a pentraxin elevated in response to inflammatory triggers and is independently associated with cardiovascular risk. For example, CRP levels are elevated in more than 65% patients suffering from unstable angina[21, 26, 27]. Similarly, levels of serum IL-6, which induces CRP-expression, have also been directly associated with CVD in epidemiological studies[28, 29]. Moreover, Mendelian randomization analyses of 40 studies including up to 133,449 individuals have identified that a polymorphism in the IL-6 receptor signaling pathway (rs7529229), which results in reduced plasma CRP levels, is associated with a decreased risk for coronary heart disease events[29, 30]. Besides CRP and IL-6 as circulating inflammatory biomarkers, pentraxin 3 (PTX3) has also been implicated in CVD, and it has been suggested that levels of PTX3 reflect local inflammation in atherosclerotic lesions more accurately than CRP [31]. Furthermore, increased serum concentration of serum amyloid A (SAA), IL-1R antagonist as well as soluble adhesion molecules have been identified as independent predictors of coronary heart disease[21, 32].

Importantly, GWAS have identified the chromosome region 9p21 as a hotspot locus for CVD risk[33, 34] and recently, in another large-scale study cyclin-dependent kinase inhibitor 2B (CDKN2B) within the 9p21 region was identified as the gene with the highest association with CVD risk[35]. CDKN2B has been investigated by Kojima *et al*. with respect to its role in atherogenesis. Interestingly, deficiency of CDKN2B in high-fat diet-fed ApoE$^{-/-}$ mice resulted in increased atherosclerosis with more complex plaques as a result of impaired apoptotic cell clearance[36]. Another candidate gene relevant to the inflammatory process of atherogenesis that was identified by GWAS encodes for the chemokine C-X-C motif ligand 12 (CXCL12), which is the ligand for C-X-C chemokine receptor type 4 (CXCR4) and is involved in neutrophil egress from bone marrow but also regulates neutrophil recruitment to atherosclerotic lesions[37].

However, identified SNPs have been associated with reduced as well as increased plasma levels of CXCL12, therefore suggesting both pro- and anti-atherogenic roles for this chemokine.

Major insights into the molecular and cellular events that drive the inflammatory response in atherosclerosis come from a plethora of experimental studies in the past years regarding the involvement of innate and adaptive immunity in atherosclerosis[8, 9, 38, 39]. These studies suggest the recognition of disease-specific antigens for the involvement of both arms of immunity as critical modulators of atherogenesis.

1.1.2.1. Antigens in atherosclerosis

A number of antigens have been identified as targets of disease-relevant immune-inflammatory responses. These include bacterial and viral antigens, though little experimental support exists for a causative involvement of infectious agents[40, 41]. Other potential antigens are products of tissue injury, such as heat shock proteins, beta2glycoprotein1 (β2GP1), which is a cofactor for cardiolipin as autoantigen, and advanced glycation-end (AGE) products[9, 19]. Major efforts have focused on the identification of antigens derived from LDL and its major protein component, ApoB-100, and several ApoB-100 peptides have been described as triggers of innate immune responses and potential antigens for B and T cells[42-44]. These peptides, such as the recently identified ApoBDS-1, may act in concert with OxLDL as key mediators of inflammation in atherosclerosis.

Most evidence supports a role for epitopes that are generated when LDL is oxidized. Both enzymatic and non-enzymatic oxidation of LDL results in the generation of structural changes that lead to the formation of neo-epitopes on the surface of OxLDL[45]. For example, when the oxidation-prone sn-2 polyunsaturated fatty acid of phosphatidylcholine undergoes oxidation, several highly reactive breakdown products, such as malondialdehyde (MDA) with its many complex condensation products, 4-hydroxynonenal (4-HNE), and the remaining “core aldehyde” 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC) are formed[46]. In turn, these aldehydes form covalent adducts with the amino groups of proteins and lipids resulting in the formation of oxidation-specific epitopes (OSE) that are recognized by specific immune responses in a hapten-specific manner[47]. Notably, OSE are also formed on the surface of dying cells and microparticles, which are circulating extracellular vesicles shed from dying or activated cells[48]. In this latter context OSE act as tags that identify cellular waste to house-keeping

functions of immunity, e.g. the clearance of dying cells. As both OxLDL and cellular debris accumulate in atherosclerotic lesions, the presence of the same OSE on both moieties also offers an explanation for the involvement of these specific immune responses in atherosclerosis.

1.1.3. Role of innate immunity in atherosclerosis

Innate immunity mediates fast and blunt responses to protect the host by providing the first line of defense against instigating stimuli. Its responses are directed at the recognition of specific molecular patterns that signal "danger" to the host. In the case of microbes, these are termed pathogen-associated molecular patterns (PAMPs)[49]. However, endogenous self-molecules can also become dangerous to the host, e.g. subsequent to tissue damage and/or excessive cell death, and also need to be sensed. Such "altered" self-molecules are recognized as damage-associated molecular patterns (DAMPs) by components of innate immunity[49]. Their recognition ensures swift responses to eliminate either microbial or endogenous antigens in order to prevent the negative consequences of tissue damage[50]. Innate immune responses involve a variety of humoral and cellular germ-line encoded pattern recognition receptors (PRRs), which are highly conserved and limited in their variability and specifically recognize PAMPs and DAMPs[51, 52]. One prototypical example for a PAMP represents lipopolysaccharide (LPS), which binds to the toll-like receptor-4 (TLR-4), a prototypic cell surface PRR. Of note, PRRs also exist as soluble form, such as CRP which binds to PC conjugated to the lipoteichoic acid present on capsular polysaccharides of S. pneumoniae[51]. Representative for many PRRs, TLRs sense not only microbial PAMPs, such as LPS, but also DAMPs, including heat-shock proteins (HSP) and heparan sulfate[49, 53]. Once they bind their cognate antigens, PRRs signal or phagocytose, and thereby play a major role in initiating the host response against dangerous stimuli[49]. Upon engagement of TLRs with their specific PAMP or DAMP, the inflammatory response gets amplified by the activation of transcription factors, such as NF-κB, which results in the secretion of pro-inflammatory cytokines and induction of co-stimulatory molecules[54]. Thus, together with the chemokines and cytokines that are released when PAMPs or DAMPs are sensed, PRRs orchestrate an inflammatory response to eliminate the inciting trigger until complete resolution. Persistent stimulation and/or impaired resolution of innate immune responses will result in chronic inflammation, as it is observed in atherosclerosis[55].

Several PRRs have been implicated in the development of atherosclerotic lesions, and many of them specifically recognize OSE, which have been identified as major DAMPs of innate immunity[56, 57]. OSE have been shown to be pro-inflammatory by activating endothelial cells leading to the up-regulation of adhesion molecules and by inducing chemokine and cytokine secretion by monocytes/macrophages[58]. It is well established that OxPL upregulates the expression of a plethora of chemokines, such as MCP-1, IL-8, IL-6 and MIP-1α[58]. Notably, the chemokines MCP-1 (CCL2) and IL-8 (KC) have been shown to play major roles in promoting atherosclerosis[59-61]. In addition, we have recently demonstrated that a subset of circulating microparticles, which have been shown to be elevated in CVD patients[62], carry OSE that have the capacity to induce IL-8 chemokine secretion by monocytes [48]. Thus, OSE play an important role in the initiation phase of atherosclerosis.

Lesional macrophages sense DAMPs via TLRs, and experimental studies in TLR-deficient atherosclerosis-prone mice have shown that TLRs play an essential role in the progression of atherosclerosis [63-65]. Initially, the involvement of TLRs in atherosclerosis stems from studies using mice deficient in MyD88, which is the intracellular adaptor protein for TLR signal transduction. MyD88-deficiency in Apolipoprotein E-deficient ($ApoE^{-/-}$) mice resulted in diminished atherosclerosis[66]. TLR-2 has been shown to play a pro-atherogenic role, as deficiency of TLR-2 in low-density lipoprotein receptor-deficient ($Ldlr^{-/-}$) mice on cells, although not of bone marrow origin, led to reduced atherosclerosis[67]. This indicates the influence of an unknown endogenous TLR-2 agonist, which contributes to atherogenesis by the activation of TLR-2 in non-bone marrow cells, which likely include endothelial cells. However, the exact ligand that is recognized by TLR-2 in atherosclerotic lesions remains elusive. Deficiency of TLR-4 also results in reduced atherosclerosis in cholesterol-fed $ApoE^{-/-}$ mice[66, 68]. The involvement of TLR-4 in lesion progression is explained by its ability to mediate pro-atherogenic responses of endothelial cells and macrophages to various OSE[69, 70]. For example, oxidized cholesterol-esters that are enriched in minimally modified LDL as well as the oxidized phospholipid oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (OxPAPC) activate macrophages via TLR-4 to secrete pro-inflammatory chemokines and cytokines, such as CXCL2 and IL-6, respectively[71, 72]. Notably, TLR-4 has been shown to heterodimerize with TLR-6 in the response to OxLDL[73]. Both the OxLDL-induced activation and chemokine expression of Gro1-α, MIP-2 and RANTES of macrophages have been shown to require a heterodimer of TLR-4/TLR-6 in cooperation with the scavenger receptor CD36, which mediates binding and uptake of OxLDL[73]. Interestingly, epidemiological studies in humans have identified that a polymorphism in TLR-4

(Asp299Gly), which is associated with decreased levels of pro-inflammatory cytokines as well as acute phase proteins and soluble adhesion molecules, and is associated with a reduced risk of carotid artery disease (CAD)[74]. However, this could not be confirmed by another study[75].

Other TLRs have also been implicated in the inflammatory response in atherosclerosis. For example, TLR-3, which is located in the endosome and senses dsRNA, as well as TLR-7, which senses ssRNA, have both been implicated as atheroprotective in studies using the $ApoE^{-/-}$ mouse model[76, 77]. Recently, a study in TLR-$9^{-/-}$$ApoE^{-/-}$ mice has revealed an atheroprotective role for TLR-9, which is located in the endosome and recognizes bacterial DNA via CpG[78]. TLR-9-deficient $ApoE^{-/-}$ mice fed an atherogenic diet showed accelerated atherosclerosis, which is mediated by $CD4^+$ T cells as depletion of those cells resulted in attenuation of atherosclerosis.

In conclusion, TLRs are critically involved in the inflammatory response in the artery wall that is characterized by the secretion of specific cytokines and chemokines, which is reviewed in detail elsewhere[8].

Another family of PRRs, scavenger receptors (SRs) have a key function in atherosclerosis, as they are responsible for the uncontrolled uptake of OxLDL, but not native LDL, resulting in the formation of foam cells - a critical pathological event in atherogenesis. A variety of SRs have been identified, such as CD36, scavenger receptor A (SRA)-1 and SRA-2, SR-BI, MARCO, LOX-1[56, 79, 80]. Of these, CD36 and SRA-1 have been found to be responsible for nearly 90% of OxLDL-uptake by macrophages through the recognition of different OSE in OxLDL[81, 82]. In case of CD36, oxidized phospholipids mediate binding of OxLDL via two different motifs - the PC head group on the one hand and the oxidized sn-2 fatty acid on the other hand[83, 84]. Among all SRs, CD36 and SRA are most prominently investigated in foam cell formation. Initial studies have shown that both CD36 and SRA are important for the formation of foam cells, as peritoneal macrophages isolated from SRA- and CD36-deficient mice account for 90% of modified LDL uptake[85]. This would suggest that genetic deletion of these SRs in mice should ameliorate atherosclerosis. However, *in vivo* studies showed mixed results. Initial studies by Febbraio *et al*., have revealed a pro-atherogenic role, as $CD36^{-/-}$$ApoE^{-/-}$ mice were protected from atherosclerosis[86]. A bone marrow transplantation study of CD36-deficient macrophages into $ApoE^{-/-}$ mice performed by the same authors also resulted in markedly diminished atherosclerosis, thereby establishing a pro-atherogenic role of CD36 on macrophages[87]. Another study investigating high-fat diet-fed $ApoE^{-/-}$ mice deficient in CD36 and SRA showed increased atherosclerosis despite a severe reduction of foam cells in peritoneal macrophages *in*

vivo, thereby suggesting a different mechanism of lipid deposition in the artery wall[88]. Moreover, combined deficiency of both CD36 and SRA resulted in no further reduction of atherosclerosis compared to CD36$^{-/-}$ApoE$^{-/-}$ control mice, suggesting a limited role of SRA in atherogenesis[89]. Taken together, the role of SRs in atherosclerosis is sometimes not clearly observed in animal studies using genetic knock-out models[79, 88, 89]. However, as several SRs are responsible for uptake of OxLDL, the effect of one genetically targeted SR may be compensated by other scavenger receptors that may be expressed at different levels in different tissues. Finally, besides their role in foam cell formation, SRs have been shown to be involved in lesional macrophage proliferation[90] and in mediating pro-inflammatory responses by TLRs (see above).

One of the consequences of OxLDL uptake and foam cell formation by macrophages is the intracellular accumulation of cholesterol crystals[91]. Cholesterol crystals are found at all stages of atherosclerosis and are in particular enriched in advanced lesions, where they are thought to trigger the physical rupture of the fibrous cap[92]. However, they have recently been found to be also involved in the inflammatory response in atherogenesis through the engagement of intracellular sensing mechanism of the inflammasome. The inflammasome, which consists of an adaptor protein apoptosis-associated speck-like protein containing CARD (ASC), as a sensor molecule, connected to caspase-1, as a molecular platform for the activation of the pro-inflammatory protease, caspase-1 that leads to the release of pro-inflammatory IL-1β and IL-18[93]. Duewell *et al*. have shown that following lysosomal damage, cholesterol crystals can activate the NLRP3 inflammasome in macrophage foam cells, which leads to the activation of caspase-1 and the secretion of IL-1β in atherosclerotic lesions[91]. Consequently, atherosclerosis-prone mice that were reconstituted with bone marrow of NLRP3-deficient donors develop decreased atherosclerosis when fed an atherogenic diet[91]. As activation of the NLRP3 inflammasome by cholesterol crystals leads to the generation of IL-18, these mice also exhibited reduced IL-18 levels. Data on the role of IL-18, which is a promoter of Th1 differentiation, in atherosclerosis points towards a pro-atherogenic role. IL-18-deficient ApoE$^{-/-}$ mice showed diminished expression of IFN-γ as well as increased IgG levels and reduced atherosclerosis compared to controls[94]. Of note, IL-18$^{-/-}$ApoE$^{-/-}$ mice showed increased serum cholesterol and triglycerides compared to controls, indicating that IL-18 has also effects on lipid metabolism. On the other hand, administration of IL-18 to ApoE$^{-/-}$ mice led to increased atherosclerosis and lesional inflammation and these effects were found to be dependent on IFN-γ[95]. Recently, an additional receptor for IL-18 has been identified, which is the NaCl co-transporter (NCC or SLC12A3)[96]. The authors have demonstrated that loss of the IL-18 receptor in ApoE$^{-/-}$ mice

alone does not have any effect in atherosclerosis, whereas co-depletion of NCC reduces atherosclerosis. These data suggest that the NaCl co-transporter has the capacity to mediate the full pro-atherogenic effect of IL-18. Rajamäki *et al.* have shown that the NLRP3 activation pathway is also functional in cholesterol crystals-exposed human macrophages, thereby linking cholesterol metabolism with inflammation in the context of atherosclerosis[97]. Taken together, experimental studies in mice identify cholesterol crystal-induced inflammasome activation and products thereof as inflammatory components in atherogenesis. CD36-mediated binding of OxLDL has been found to coordinate these responses via priming which is TLR-4/6-dependent and expression of pro-IL-1β, which are the required signals for the full activation of the NLRP3 inflammasome in macrophages, as well as via endocytic uptake of OxLDL resulting in cholesterol crystal accumulation[98].

Thus, OSE represent pro-inflammatory DAMPs that engage several PRRs to promote inflammatory responses in atherosclerosis. In addition to their presence on OxLDL, OSE on microparticles and dying cells can also contribute to inflammatory responses[99]. Particularly, later stages of atherosclerosis with excessive accumulation of OxLDL are also associated with increased foam cell apoptosis[55]. In turn, uptake of apoptotic foam cells also contributes to cholesterol uptake by macrophages promoting a vicious cycle of disease promotion. Furthermore, impaired clearance of apoptotic foam cells results in their accumulation, which further renders them pro-inflammatory and contributes to the chronic inflammatory process of atherosclerosis[55]. Resolution of inflammation depends on the efficient clearance of apoptotic cells, a mechanism called efferocytosis. Efficient apoptotic cell clearance would prevent secondary necrosis thereby limiting inflammation. In fact the swift uptake of apoptotic cells has been shown to induce anti-inflammatory cytokines, such as TGF-β and IL-10, which may diminish plaque progression in certain settings, such as early stages of lesion development[55, 100]. Several studies have identified a critical role for apoptotic cell accumulation in atherogenesis. In this regard, MFGE8 (milk fat globulin E8) expressed by macrophages has been identified as a bridging molecule, which promotes the phagocytosis of apoptotic thymocytes via the interaction of phosphatidylserine on apoptotic cells and αvβ3 integrin on phagocytes[101]. Ait-Oufella *et al.* reconstituted irradiated $Ldlr^{-/-}$ mice with $Mfge8^{-/-}$ bone marrow, which were then fed an atherogenic diet and displayed systemic as well as lesional accumulation of apoptotic cells[102]. Furthermore, these mice had reduced splenic IL-10 as well as increased splenic and lesional IFN-γ accompanied by changes in suppressive functions of Tregs dependent on dendritic cells (DCs). Interestingly, mice reconstituted with $Mfge8^{-/-}$ bone

marrow also displayed increased levels circulating microparticles. Thus, Mfge8 plays a role in controlling the accumulation of apoptotic cells within atherosclerotic plaques, consequently reducing atherosclerotic lesion development. Another protein that is implicated in apoptotic cell clearance represents mer receptor tyrosine kinase (MertK). Its role in atherosclerosis has been addressed by two independent studies. One study investigated high-fat diet-fed MertK-deficient ApoE$^{-/-}$ mice and observed increased atherosclerosis accompanied by more lesional apoptotic cells and larger necrotic cores[103]. On the other hand, Ait-Oufella *et al.* reconstituted Ldlr$^{-/-}$ mice with mertk-deficient bone marrow and observed increased apoptotic cell accumulation, as well as an inflammatory plaque phenotype and enhanced atherosclerosis[104]. The classical way of complement activation has also been shown to promote the clearance of apoptotic cells. In this regard, Ldlr$^{-/-}$ mice deficient in C1q have increased lesional apoptotic cells and display larger atherosclerotic lesions[105]. Furthermore, deficiency of transglutaminase-2 (TG2), which is a regulator of protein crosslinking, in bone marrow transplanted Ldlr$^{-/-}$ mice fed an atherogenic diet has been associated with increased apoptotic cells inside lesions[106]. Collectively, these data implicate different ways of apoptotic cell disposal in the context of atherosclerosis. Notably, both TLR-4 as well as scavenger receptors have been also implicated in the recognition of microparticles and apoptotic cells[107, 108].

1.1.4. Role of the adaptive immune system in atherosclerosis

Adaptive immunity is represented by the activation of T and B cells that recognize antigens via B cell receptors (BCR; membrane bound Ig) and T cell receptors (TCRs), respectively. In contrast to innate PRRs, these receptors are a product of somatic recombination giving rise to a nearly unlimited repertoire of specificities. Following antigen encounter different responses are effected by T and B cells to eliminate antigens. These responses are under tight control by several mechanisms that prevent exaggerated or inappropriate responses, including elimination of self-reactive clones as well as the down-regulation of initiated responses. Accumulating evidence now suggests that the balance and the tight regulation is lost in atherosclerosis, resulting in pro-inflammatory responses and a failure of resolution. Histological evidence for the presence of both activated CD4$^+$ and CD8$^+$ T cells and antigen presenting cells as well as immunoglobulins in atherosclerotic plaques initiated decades of research investigating the role of adaptive immune functions in atherosclerosis[109]. In fact, clonal expansion of T cells has been demonstrated in human lesions by TCR spectratyping from coronary artery specimen as well as

in lesions at different stages in ApoE$^{-/-}$ mice[110, 111] indicating the occurrence of antigen-specific reactions inside the lesions. Moreover, plaque T cells were found to have a restricted usage of TCRs indicative of an oligoclonal expansion[112]. Moreover, T cells isolated from lesions were found to specifically proliferate in response to OxLDL when presented in an MHC-II restricted manner and immunoglobulins eluted from lesions were found to be complexed with OxLDL[112, 113]. This is complemented by an increasing number of clinical studies demonstrating a significant association of antibody titers to OxLDL and HSP60 as well as frequencies of specific T and B cell subsets with CVD[114-116].

For example, an increased frequency of CD8$^+$ T cells has been identified to be associated with a higher incidence of coronary events[117]. In addition, higher circulating levels of CD4$^+$CD28^{null} cells are associated with poor prognosis and recurrence of acute coronary syndrome[118]. Regarding the role of circulating Tregs, clinical studies have revealed conflicting results. One study has investigated circulating Tregs in patients with carotid or coronary atherosclerosis and did not find a correlation with CAD[119]. This is in contrast to previous studies, which reported that patients with acute coronary syndrome show a decreased frequency of circulating Tregs in their blood[120-122]. One may speculate that discrepancies of these analyses results from differences in the assessment of these T cell subpopulations. In contrast to T cells, only few B cells are present inside the plaques. Nevertheless, systematic analyses of GWAS as well as of gene expression data of peripheral blood from the Framingham Heart Study have causally linked B cell immune responses in CVD[123].

Thus, several lines of evidence suggest an involvement of adaptive immunity in human atherosclerosis, and the profoundly increased cardiovascular risk in patients with autoimmune diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), provides additional support to this notion.

Critical evidence for the involvement of adaptive immunity comes from models of experimental atherosclerosis. This is best documented by the fact that Ldlr$^{-/-}$ or ApoE$^{-/-}$ mice rendered lymphocyte-deficient (through deficiency in Rag1$^{-/-}$, Rag2$^{-/-}$ or SCID) develop significantly less atherosclerosis, suggesting a net pro-atherogenic effect of adaptive immunity[124-126]. However, even in the absence of adaptive immunity mice develop atherosclerotic lesions and when cholesterol levels are exceedingly high no effect of lymphocyte deficiency is observed[127]. Thus, adaptive immune functions are not required for lesion formation, but are important modulators of the disease process. Moreover, the complete absence of both T and B cells may result in the

elimination of both pro- and anti-atherogenic subsets that possess different activities in atherosclerosis. Thus, the dissection of the individual functions of different T and B cell subsets has been the focus of the past years to understand the complex interplay of adaptive immune responses in atherosclerosis as well as mechanisms by which these can affect disease acceleration in certain conditions.

1.1.4.1. T cells in atherosclerosis

$CD4^+$ Th cells have been identified as a prominent subset of atherosclerotic lesions of all stages of disease[128] (see Fig. 1). Based on the cytokines they secrete they can polarize into different subsets exhibiting different effector functions. Th1 cells, which produce pro-inflammatory cytokines, such as IFN-γ, are critical involved in the initiation and progression of atherosclerosis[129]. IFN-γ exerts pro-atherogenic activities, including the induction of adhesion molecules as well as chemokine expression by endothelial cells, the stimulation of pro-inflammatory cytokines and chemokines by macrophages, the production of reactive oxygen species as well as matrix metalloproteinases by macrophages, the inhibition of cholesterol efflux from foam cells as well as preventing collagen synthesis by vascular smooth muscle cells[130]. In addition, IFN-γ exerts its functions as activator of monocytes/macrophages as well as dendritic cells, consequently resulting in an ongoing pathogenic Th1 response[32]. Administration of IFN-γ accelerated atherosclerosis in $ApoE^{-/-}$ mice[131], whereas $ApoE^{-/-}$ or $Ldlr^{-/-}$ mice deficient in IFN-γ display reduced atherosclerosis[132, 133]. In these settings, T cell-independent IFN-γ secreted by macrophages, natural killer cells as well as vascular cells were found to be responsible for disease progression. All of these experimental studies performed point towards a role of IFN-γ in lesion promotion. Indeed, IFN-γ secreting T cells have been isolated from human plaques[134]. The prominent role of Th1 responses in atherogenesis is strongly supported by number of studies demonstrating that atherosclerosis is promoted by IFN- γ and cytokines, such as IL-12 and IL-18, which induce Th1 cell differentiation and IFN-γ secretion[135]. Moreover, impaired Th1 differentiation in mice lacking the Th1 lineage-specific transcription factor T-bet results in reduced atherosclerosis[136]. On the other hand, the role of type I interferons (including IFN-α and IFN-β) in atherosclerosis remains to be explored, as different studies have revealed both pro- as well as anti-inflammatory functions[137]. For example, IFN-α produced by aortic plasmacytoid dendritic cells (pDC) has been shown to several fold upregulate TNF-related apoptosis-inducing ligand (TRAIL) on $CD4^+$ T cells, consequently inducing the cytolytic functions for smooth muscle

cells[138]. Moreover, IFN-α has been shown to promote the uptake of OxLDL as well as foam cell formation via the upregulation of the expression of SRA[139]. Recently, more light was shed on the role of pDCs in atherosclerosis. Ldlr$^{-/-}$ mice selectively deficient for the transcription factor Tcf4, which results in loss of pDCs, were protected from atherosclerosis and displayed reduced Th1 cells[140]. Moreover, treatment of Ldlr$^{-/-}$ and ApoE$^{-/-}$ mice with IFN-β results in increased atherosclerosis by enhancing macrophage adhesion to sites of atherosclerotic plaque formation in a chemokine-dependent manner[141]. It is speculated that upregulated chemokines as well as their receptors plays a role in accelerated atherosclerosis in this model. Interestingly, IFN-β treated mice exhibited more lesional macrophages, despite no differences in total cholesterol accompanied by increased IL-10 production compared to controls. The same authors also investigated the role of the major receptor for type I IFN, which is called IFNAR1, by transplanting IFNAR1$^{-/-}$ bone marrow cells into Ldlr$^{-/-}$ mice[141]. As a result, mice exhibited markedly reduced atherosclerosis as well as decreased necrotic cores inside the lesions.

Th17 cells are the main producers of IL-17, but their role in atherosclerosis remains controversial, as both anti- as well as pro-atherogenic effects for IL-17 have been found in experimental atherosclerosis[142, 143]. However, experimental studies using IL-17 blocking antibodies or genetic deletion of IL-17A in Ldlr$^{-/-}$ as well as ApoE$^{-/-}$ mice favor a pro-atherogenic role of Th17 cells and IL-17, as this resulted in decreased atherosclerotic lesion formation, reduced numbers of lesional macrophages and ROS generation, and diminished expression of pro-inflammatory cytokines, such as TNF-α and IL-6[144-146]. In contrast, administration of IL-17 has been shown to decrease atherosclerosis[143, 147] and IL-17 deficiency accelerated atherogenesis[147], thereby also identifying atheroprotective roles for IL-17. Thus, investigations in several experimental studies of the role of Th17 cells and IL-17 in atherosclerosis requires further investigation[148].

Similarly, data on the function of Th2 cells, which are characterized by the secretion of IL-4, IL-5, and IL-13, and down-regulate Th1-mediated responses, stimulate antibody production by B cells, and promote alternative macrophages activation[149] provide mixed results. IL-4 has been suggested to be pro-atherogenic, although Ldlr$^{-/-}$ mice deficient in IL-4 did not shown any effect in atherosclerosis in one study[150], previous work by the same group has indeed demonstrated decreased atherosclerosis[151, 152]. In contrast, a protective role for IL-5 and IL-13 has been established in experimental atherosclerosis[153]. We have shown that IL-5 possesses the capacity to stimulate atheroprotective OxLDL-specific natural IgM and that cholesterol-fed Ldlr$^{-/-}$ mice reconstituted with IL5-deficient bone marrow develop increased atherosclerosis[154]. On the other

hand, IL-13 protects from atherosclerosis through the promotion of a favorable plaque phenotype and the induction of anti-inflammatory alternatively activated macrophages[155]. However, these protective Th2 cytokines are also secreted by type 2 innate lymphoid cells[156], suggesting these effects to be independent of Th2 cells.

Although $CD8^+$ cytotoxic T cells are less abundant in atherosclerotic plaques, they may have a role particularly in advanced atherosclerosis. Depletion studies using monoclonal antibodies for CD8α and CD8β in high-fat diet fed $ApoE^{-/-}$ mice have shown that $CD8^+$ cells mediate pro-inflammatory pro-atherogenic effects[157]. To gain mechanistic insights, Kyaw *et al.* performed adoptive transfer experiments of $CD8^+$ T cells deficient in the cytolytic enzymes perforin and granzyme-B into lymphocyte-deficient $ApoE^{-/-}$ mice, which did not increase atherosclerosis. These enzymes mediate target cell lysis upon the activation of $CD8^+$ T cells into cytotoxic T cells and therefore promote apoptosis-induced inflammation and necrosis[158]. In addition, the authors have shown that also TNF-α is implicated mechanistically, as $CD8^+$ T cells are potent producers of this cytokine, which is an important inflammation modulator. These data provide experimental evidence for a pro-atherogenic role for $CD8^+$ T cells via perforin and granzyme-B mediated cytotoxicity as well as TNF-α promoted inflammatory mechanisms.

T cell responses are tightly regulated by regulatory T cells (Tregs) - most prominently Foxp3 expressing $CD4^+CD25^+\alpha\beta TCR^+$ cells. Notably, a series of studies have identified a clear atheroprotective role for Tregs, which suppress and counterbalance immune responses by regulating co-stimulation or secreting specific cytokines, including the atheroprotective cytokines IL-10 and TGF-β. Depletion studies by anti-CD25[159] or immunization with Foxp3-transfected DCs[160] have demonstrated an atheroprotective role of Tregs. Moreover, direct transfer experiments of Tregs have demonstrated that these can limit T cell responses and lead to diminished atherosclerosis[161, 162]. In addition, blocking or deficiency of TGF-β and IL-10 has been shown to increase atherosclerosis[163-166]. It can be speculated that enhancing the suppressive functions of Treg may provide an attractive method to inhibit pro-atherogenic responses of both $CD4^+$ and $CD8^+$ T cells.

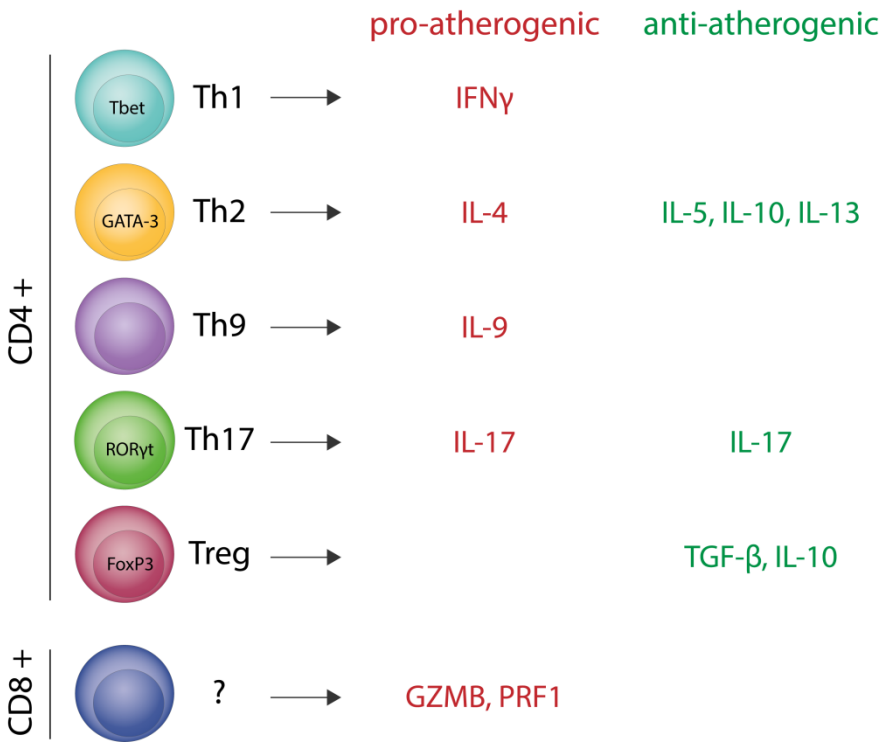

Fig. 1: The role of T cells in atherosclerosis. Th1 cells, which differentiate under the control of the transcription factor T-bet, secrete IFN-γ and promote atherosclerosis. Th2 cells are the producer of IL-4, IL-5, IL-10 and IL-13, which have shown to possess different roles in atherosclerosis. The recently identified Th9 cells produce IL-9, which is suggested to play a pro-atherogenic role. However, a direct implication of IL-9 as well as Th9 cells in atherosclerosis has to be elucidated. Th17 cells produce IL-17, but the exact role in the context of atherosclerosis is controversial. Tregs are the producers of the cytokines IL-10 and TGF-β, which are atheroprotective. $CD8^+$ T cells have been suggested to be pro-atherogenic via a mechanism involving perforin- and granzyme-B-mediated apoptosis.

1.1.4.2. B cells in atherosclerosis

Several studies over the past 15 years have shown that B cells are key modulators of hypercholesterolemia-induced inflammatory milieu[191], despite the fact that they are present in low numbers in both murine and human atheromas[167-169]. GWAS data support a role of B cells in human atherosclerosis by involving proliferation and activation status of B cells as important factors in CVD risk[123]. In agreement with this, increased numbers of activated $CD19^+CD86^+$ B cells were found to be associated with increased risk for stroke[170].

In support of the human data, a large set of experimental studies has investigated the role of B cells in atherosclerosis. Caligiuri *et al*., was the first to address how B cells affect experimental atherosclerosis by investigating the effect of adoptive transfers of B cells into splenectomized atherosclerosis-prone ApoE$^{-/-}$ mice[171]. The authors reported that B cells isolated either from wild type or ApoE$^{-/-}$ donors reversed the splenectomy-induced accelerated atherosclerosis. Notable, the protective effect was even more profound in the splenectomized recipients that received B cells from ApoE$^{-/-}$ donors. These data were in line with a study showing that lethally irradiated Ldlr$^{-/-}$ mice that were injected with bone marrow isolated from B cell deficient (μMT) donors developed increased atherosclerosis compared to recipients that received wild-type bone marrow[172].

Although these studies indicate an atheroprotective role of B cells, one has to keep in mind that B cells are a very heterogeneous population including several different subsets with sharply different properties with respect to their activation, immunoglobulin profile secretion as well as differentiation pathways. B cells are divided in two large subsets, the conventional B2 and B-1 populations. For example, B2 cells (which include follicular (FO) and marginal zone B (MZB) cells) are short lived, they are derived from the bone marrow and they become activated mainly in a T cell dependent manner. On the other hand, B-1 cells have fetal liver origin and they produce pre-existing, germline encoded natural IgM antibodies in absence of T cell help[173, 174]. Thus, dissecting the role of individual not only subsets but also activation pathways is important in order to translate these findings into the clinic (see Fig. 2).

Along these lines, treatment of hypercholesterolemic ApoE$^{-/-}$ and Ldlr$^{-/-}$ mice with the B cell depleting anti-CD20 antibody, which preferentially depletes B2 cells and results in a strong reduction of total IgG titers, while it largely preserves B-1a cells and natural IgM, confers an atheroprotective effect[175, 176]. The atheroprotective effect of anti-CD20 treatment was attributed to enhanced Th17 responses as anti-IL17 antibody treatment abrogated the atheroprotective effect of anti-CD20 injections. These data suggest that B2 cells exhibit a pro-atherogenic effect. This conclusion is supported by the finding that adoptive transfer of splenic B2 cells into lymphocyte-deficient Rag2$^{-/-}$γ-chain$^{-/-}$ApoE$^{-/-}$ or μMT/ApoE$^{-/-}$ recipients enhanced atherosclerosis[176]. It is important to note that the latter data imply that B2 cells are also able to aggravate atherosclerosis in absence of T cells; however the mechanisms by which they do so require further investigation. Furthermore, studies that investigate the role of the B cell activating factor (BAFF) system in atherosclerosis also suggest a pro-atherogenic role for B2 cells. BAFF binds to BAFF receptor (BAFFR) and thereby facilitates the survival of B2 cells, while of note

this pathway is indispensable for the survival of B-1 cells[177]. Therefore, BAFFR-deficient animals lack B2 cells[178]. In line with this, Baffr$^{-/-}$ApoE$^{-/-}$ mice, lethally irradiated Ldlr$^{-/-}$ that were transplanted with BAFFR-deficient bone marrow, and ApoE$^{-/-}$ mice treated with an anti-BAFFR blocking antibody lack B2 cells and develop decreased atherosclerosis[179-181]. However, the pro-atherogenic role of B2 cells has been challenged by Doran *et al*., who observed decreased atherosclerosis in μMT/ApoE$^{-/-}$ mice upon adoptive transfer of splenic B2 cells isolated from ApoE$^{-/-}$ donors[182]. Thus, further studies are required to pinpoint the exact role of B2 cells in atherosclerosis.

In contrast, the data on the role of B-1 cells suggest a protective role in atherosclerosis. B-1 cells predominantly localize in the peritoneum and pleural cavities and are divided in B-1a and B-1b cells[173]. Evidence regarding the atheroprotective role of B-1a cells comes from Kyaw *et al*., who showed that adoptive transfer of natural IgM secreting B-1a cells (in contrast to sIgM$^{-/-}$ B-1a cells) into splenectomized ApoE$^{-/-}$ mice, which develop accelerated atherosclerosis and display 50% reduced plasma IgM, reversed the splenectomy induced accelerated atherosclerosis[183]. Moreover, a recent report by Rosenfeld *et al*. demonstrated that adoptive transfer of B-1b cells into Rag1$^{-/-}$ApoE$^{-/-}$ mice resulted in decreased lesion formation compared to PBS injected controls[184]. The limitation in both above mentioned studies is that they address the role of the B-1 cell subsets in specific contexts (splenectomy and lymphocyte deficiency) and do not allow conclusions for the role of these cells in intact conditions. For example, a recently identified B-1a derived cell subset, the innate response activator B cells (IRA B cells)[185] have been shown to expand in the spleen of hypercholesterolemic mice and interestingly they aggravate atherosclerosis, presumably via enhancing Th1 responses and anti-OxLDL IgG_{2c} production[186]. These data suggest that increasing the B-1a numbers could result in enhanced IRA B cell generation and thus more atherosclerosis. Therefore studies addressing the role of B-1 cells in an intact immune system are of great interest in order to gain confidence regarding their atheroprotective role.

Finally, the potential role of B regulatory cells (Breg) in atherosclerosis has recently been investigated. Breg are characterized by their increased production of the atheroprotective cytokine IL-10[187, 188]. Strom *et al.*, recently demonstrated that Breg increased in the lymph nodes of hypercholesterolemic ApoE$^{-/-}$ mice. Interestingly adoptive transfer of lymph node-derived B cells into ApoE$^{-/-}$ recipients resulted in an atheroprotective effect that was dependent on the ability of these cells to produce IL-10[189]. In line with this, the authors also reported that purified Breg (defined as CD21^{high}CD23^{high}CD24^{high}) from the lymph nodes of ApoE$^{-/-}$ donors resulted

also in atheroprotection. While these data suggest that Breg confer atheroprotection via IL-10 secretion, Sage *et al*., reported that B cell derived IL-10 does not affect atherosclerotic lesion formation[190]. Taken together, further studies are also required to elucidate the role of Breg in atherosclerosis.

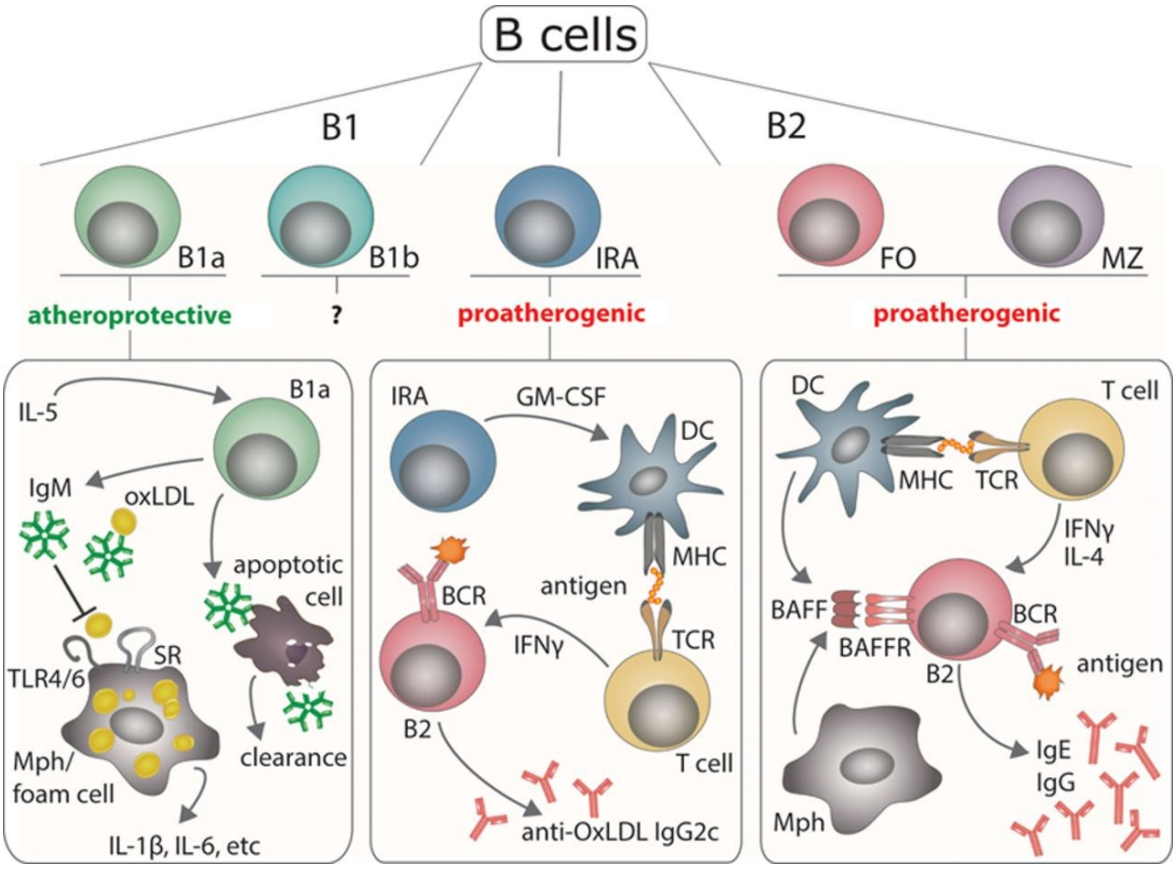

Fig. 2: B cell subsets and their role in atherosclerosis. B cells can be subdivided into the major subsets B-1 and B2 cells. B-1 cells can be further distinguished into B-1a and B-1b subsets, of which both secrete natural IgM and have been shown to be atheroprotective. In contrast to this, B2 cells which comprise of FO and MZB cells have been suggested to be pro-atherogenic - however the mechanisms are not yet clear. IRA B cells, which are a subset of B-1a cells and secrete GM-CSF, have been shown to promote atherosclerosis via the expansion of anti-OxLDL IgG2c antibodies. BAFFR, B cell activating factor receptor; BCR, B cell receptor; GM-CSF, granulocyte macrophage colony-stimulating factor; MHC, major histocompatibility complex; Mph, macrophage. Adopted with permission from Tsiantoulas *et al*.[191]

1.1.4.3. Humoral immunity in atherosclerosis

An important role for immunoglobulins in atherosclerosis is supported by both epidemiological and experimental studies[192]. Epidemiological studies - though not consistently - have reported a positive association of anti-OxLDL or anti-Hsp65 IgG antibodies and CVD adverse effects[153, 193]. Experimental studies mainly addressing the effect of immunization against prominent antigens of atheromas such Hsp65 and OxLDL have overall suggested a pro-atherogenic role for IgG (see Fig. 3). For example, immunization of normocholesterolemic rabbits or chow diet-fed $Ldlr^{-/-}$ mice with Hsp65 accelerated atherosclerosis[194]. These data are in line with a study by George *et al.* who infused IgG preparations from Hsp65-immunized mice into chow-fed $Ldlr^{-/-}$ mice resulting in enhancement of fatty streak formation[195]. On the other hand, immunization of $Ldlr^{-/-}$ WHHL rabbits with homologous MDA-LDL resulted in the strong induction of MDA-LDL specific IgG titers and decreased atherosclerosis compared to controls[196]. Similar data were obtained from $ApoE^{-/-}$ or $Ldlr^{-/-}$ mice that were immunized with MDA-LDL [154, 197-200]. An explanation for these discrepancies could be that IgG antibodies consist of different subclasses (humans: IgG1, IgG2, IgG3, IgG4; mice: IgG1, IgG2a/c, IgG2b, IgG3) and therefore activate different families of Fcγ receptors (activating or inhibitory)[201, 202]. For example, $ApoE^{-/-}$ mice lacking the Fcγ-chain and thus lack all activating receptors, but still express the inhibitory receptor FcγRIIB, develop decreased atherosclerosis[203]. In line with this, $ApoE^{-/-}$ mice that lack the inhibitory FcRγIIB receptor display increased atherosclerosis[204]. Despite its particularly low abundance in plasma, IgE seems to possess an important role in the development of atherosclerosis (see Fig. 3). Epidemiological studies have reported a positive association between plasma IgE levels and CVD risk[192, 205]. Importantly, IgE antibodies have been recently shown to promote and correlate with active SLE[206]. The latter is particularly important as SLE patients are characterized by premature atherosclerosis development and high prevalence of myocardial infarction[207]. Recently, a study by Wang *et al.* has suggested a disease promoting role for IgE in experimental atherosclerosis by examining atherosclerosis-prone mice that lack the high affinity receptor for IgE (FcεRI). These mice developed significantly reduced both plaque size and complexity. Moreover, the authors reported that IgE were able to promote macrophage and smooth muscle cell activation and/or death[208]. However, it remains unknown what the endogenous antigens that IgE recognize are and if antigen-binding is required.

A major protective role in atherosclerosis has been attributed to IgM antibodies (see Fig. 3). The secreted form of IgM antibodies - in contrast to IgG and IgE - consist of a pentameric structure.

The vast majority of IgM antibodies (~80%) are produced in absence of any T cell or cognate help. These natural antibodies are germline encoded and appear very early in life[173, 192, 209]. We have previously shown that >35% of IgM recognize different OSE[210]. Previous studies have shown that OSE-specific natural IgM have the capacity to block OxLDL uptake and promote the clearance of apoptotic cells[211]. For example, the T15/E06 clone, which binds to PC of oxidized phospholipids, has been shown to block the recognition of POVPC by the scavenger receptor CD36 [212]. Moreover, the OSE-specific clone NA-17, which recognizes MDA, has been shown to promote uptake of apoptotic cells by macrophages[210, 213]. These properties of natural IgM are particularly important in counteracting atherosclerosis progression. In line with this, we have shown that cholesterol-fed atherosclerosis-prone mice immunized with heat-killed S. pneumoniae, developed strongly increased T15/E06 antibodies and decreased atherosclerosis[214]. These results were in agreement with a study by Faria-Neto *et al.*, who reported reduced vein graft atherosclerosis in ApoE$^{-/-}$ mice that received T15/E06 IgM antibodies[215]. Further evidence on the atheroprotective role of T15/E06 comes from our studies in which immunized mice with MDA-LDL developed high titers of T15/E06 antibodies in an IL-5 dependent manner and decreased atherosclerosis[154]. These data are also supported by epidemiological studies showing an inverse correlation of anti-OxLDL IgM plasma levels and CVD risk[192]. Of great interest, similar data were reported for SLE patients. For example, it has been shown that SLE patients display an inverse association between low anti-PC IgM levels (even when normalized to total IgM) and atherosclerotic plaque incident[216, 217]. These studies are particularly interesting considering the premature atherosclerosis development in SLE patients and point to an important immunomodulatory role of anti-OxLDL IgM. Therefore, identifying the crucial epitopes on OxLDL as well as the right vaccination approach that favors the expansion of such atheroprotective IgM would be of particular interest in the prevention of atherosclerosis. For example, we have recently identified peptide mimotopes of MDA that are recognized by specific human IgM antibodies[218].

The studies above suggest a protective role of increased (above physiological) levels of IgM. These protective effects are mediated via preventing foam cell formation and promotion of apoptotic cell clearance. Interestingly, reduced levels of IgM result in aggravated atherosclerosis. Lewis *et al.*, have shown that mice deficient in secreted IgM develop accelerated atherosclerosis[219]. In agreement with this, Kyaw *et al.*, reported that the accelerated atherosclerosis in ApoE$^{-/-}$ mice upon splenectomy, which also results in 50% reduced plasma

IgM, is entirely reversed by adoptive transfer of IgM secreting B-1a cells (reconstitution of plasma IgM) compared to mice that received sIgM$^{-/-}$ B-1a cells[183]. However, both studies provide no evidence that the IgM deficiency or the protective effect of IgM normalization is attributed to changes in apoptotic cell or OxLDL clearance. These data suggest that the pro-atherogenic effect of deficiency or reduced levels is mediated via different - currently unknown – mechanisms. Notably, secreted IgM deficiency in lupus-prone mouse model has been shown to accelerate autoantibody formation, enhanced glomerulonephritis and decreased survival[220]. Taken together, one may speculate that low IgM levels could also be a risk factor for CVD in SLE patients.

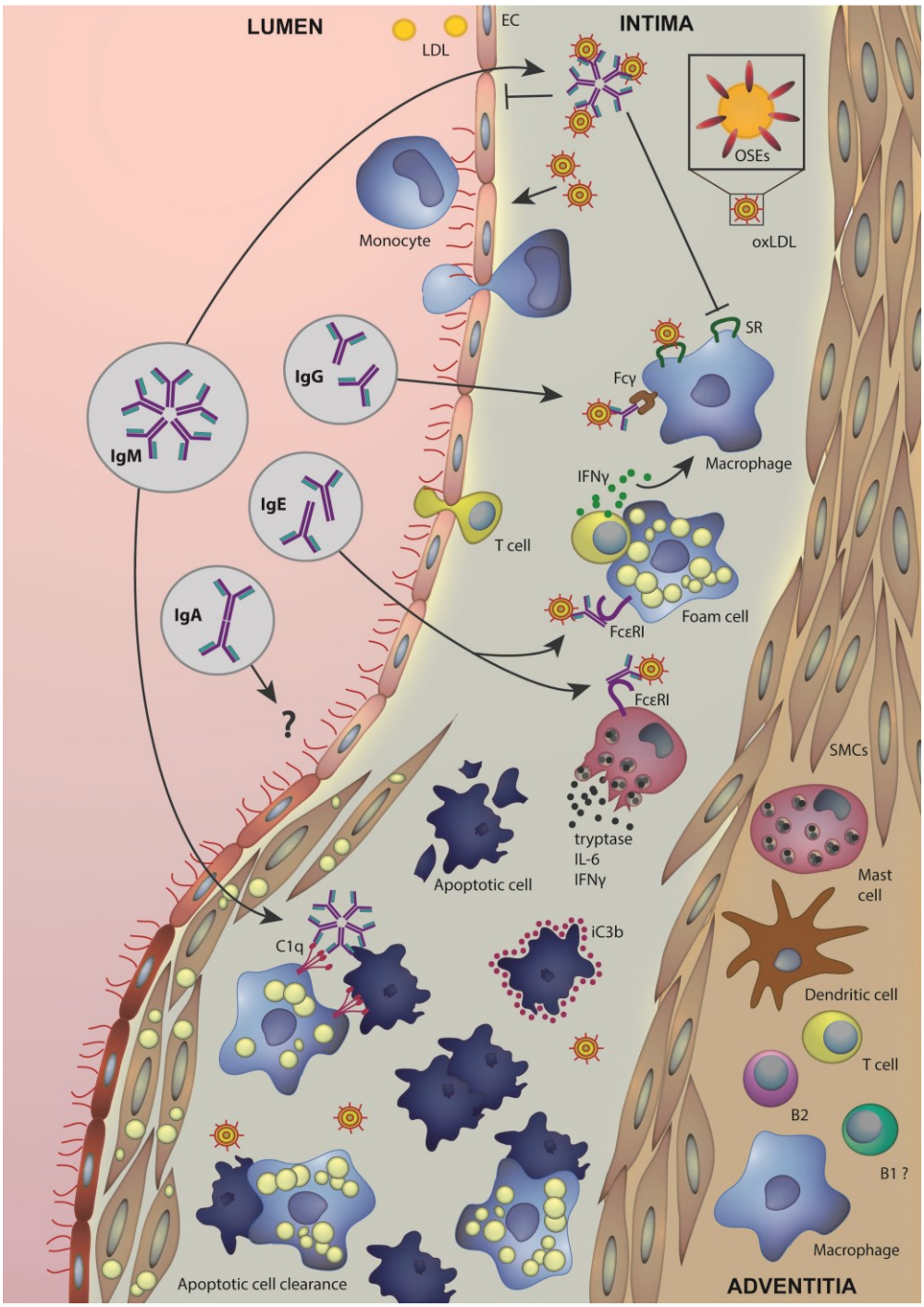

Fig. 3: Immunoglobulins and their role in atherosclerosis. A variety of immunoglobulins are present in atherosclerotic plaques. For example, HSPs and OxLDL are prominent antigens for these immunoglobulins. IgM antibodies exert a variety of atheroprotective functions, such as the neutralization of pro-inflammatory properties of OxLDL, inhibition of the uptake of OxLDL by macrophages and promotion of the clearance of apoptotic cells. IgG antibodies specific for OxLDL have been suggested to be pro-atherogenic via the activation of macrophage Fcγ-receptors. IgE antibodies have been shown to promote plaque destabilization via the activation of mast cells and macrophages via FcεRI engagement. The role of IgA antibodies in atherosclerosis remains elusive. Adopted with permission from Tsiantoulas *et al.*[193]

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The introductory chapter “ATHEROSCLEROSIS” is reprinted from the following book chapter: **“**Immune-mediated accelerated atherosclerosis.”

Gruber S, Tsiantoulas D, Binder CJ

The Heart in Rheumatologic, Inflammatory and Autoimmune Diseases: Pathophysiology, Clinical Aspects and Therapeutic Approaches

Elsevier (Book chapter, in press)

1.2. Immunometabolism

The emerging field of immunometabolism describes processes, in which metabolism and immunity regulate each other's function (Murray et al, 2015). Proper control of this interaction is increasingly recognized as essential for the maintenance of homeostasis and health. Indeed, there is growing evidence that specific metabolic perturbations result in the accumulation of dying cells and/or metabolic by-products that trigger tissue inflammation if not efficiently removed (Tabas, 2010). Persistence of this inflammatory response and its impaired resolution paves the way for chronic inflammation, which has been shown to propagate associated pathologies such as vascular and hepatic inflammation. Thus, there is growing interest in identifying mechanisms that enhance the immune system's capacity to prevent endogenously triggered inflammation and/or promote its resolution.

1.3. Hepatic inflammation and its mechanistic similarities to atherosclerosis

The term non-alcoholic fatty liver disease (NAFLD) represents a variety of liver diseases with different shades of severity. This ranges from pure steatosis to non-alcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma (Parekh & Anania, 2007). NASH can be seen as the hepatic event of the metabolic syndrome and is characterized by the accumulation of triglycerides in the liver combined with hepatic inflammation. Steatosis is characterized as the benign and reversible stage, while the other stages are characterized by the presence of inflammation (Musso et al, 2016). As a consequence, persistent inflammation leads to further liver damage and ultimately leading to irreversible and severe dysfunction of the liver – as seen in cirrhosis and liver cancer.

The mouse model deficient for the low-density lipoprotein receptor (*Ldlr*$^{-/-}$), which is an important gene in lipid metabolism, is a widely used standard mouse model in atherosclerosis research (Bieghs et al, 2012c). It has been shown that deficiency of the LDL receptor results in profoundly increased cholesterol levels in mice when fed a high-fat, high cholesterol diet. The majority of cholesterol was present in the IDL/LDL fraction, whereas a small part was confined to the VLDL fraction (Ishibashi et al, 1993). In contrary to the lipid profile in normal mice, which is characterized by the majority of the cholesterol being present in the HDL fraction, this profile is more comparable to the lipoprotein profile observed in humans, in which cholesterol is mainly present in the LDL fraction. In recent years, NASH research has made use of this mouse model

as well, as it serves as a physiological mouse model to study the progression of hepatic inflammation and early phases of NASH (Bieghs et al, 2012c).

Atherosclerosis and NASH can be seen as potential consequences of the metabolic syndrome, which is furthermore associated with abdominal obesity, inflammation and insulin resistance or diabetes. In this regard, both environmental as well as genetic factors play an important role in the development of the metabolic syndrome (Sarafidis & Nilsson, 2006). Interestingly, several studies have indicated an association between NASH and an elevated risk for developing CVD (Bieghs et al, 2012a). However, only a few studies have directly shown a contribution of liver inflammation to atherogenesis. For example, increased levels of the CVD risk factors fibrinogen, plasminogen activator inhibitor-1 (PAI-1) and CRP have been detected in NASH patients and show a correlation with histological severity which is independent of traditional risk factors (Targher et al, 2008; Yoneda et al, 2007).

Overall, these studies propose that NASH plays a role in further promoting chronic inflammation via the secretion of the above mentioned pro-inflammatory factors by the liver. Moreover, elevated myeloperoxidase (MPO) activity is seen in NASH patients and has been suggested to contribute to lipid peroxidation and promotes the progression of NASH and atherosclerosis (Ikura et al, 2006; Rensen et al, 2009; Wong et al, 2009). Even though atherosclerosis and NASH are two separate consequences of the metabolic syndrome, recent insights have revealed that some underlying mechanisms that lead to the inflammatory response are similar in both pathologies (Bieghs et al, 2012a).

In both diseases, macrophages and cholesterol play a central role. As mentioned previously in this thesis, macrophages have an important role in atherogenesis by communicating inflammatory signals between metabolic tissues as well as in scavenging modified lipids. The recruitment of monocytes and the maturation into macrophages as well as the uptake of OxLDL via scavenger receptors inside the vessel wall consequently lead to the formation of so called foam cells inside the atherosclerotic plaque (Moore & Freeman, 2006). Of note, the accumulation of lipid-laden macrophages has been observed in the liver during NASH (Wouters et al, 2008). In more detail, when mice were fed a high-fat diet foamy Kupffer cells were observed in the livers of these mice (Wouters et al, 2008). These Kupffer cells represent hepatic macrophages which express scavenger receptors and are capable of uptake of modified lipoproteins. Targeted deletion of scavenger receptor A and CD36 in mouse macrophages leads to decreased hepatic inflammation (Bieghs et al, 2012c; Bieghs et al, 2010). In analogy, it has

been shown that deficiency of scavenger receptors in macrophages in atherosclerotic mice results in diminished atherosclerosis (Moore & Freeman, 2006).

Collectively, atherosclerosis and NASH can be seen as pathologies with similar underlying mechanisms in which macrophages and cholesterol play a central role as a driver of the inflammatory response.

1.4. Siglec-G

1.4.1. Characterization, expression, structure and biochemical function

Siglec-G is a member of the CD33-related Siglecs, consisting of five immunoglobulin-domains. The human ortholog of Siglec-G is called Siglec-10 and has been determined by sequence similarity and analogy of the gene locus structure (Angata et al, 2004).
The intracellular part of Siglec-G contains three signal-motifs which might be responsible for the intracellular signal transduction (Crocker et al, 2007). These motifs bear a Grb2-binding motif, ITIM (immunoreceptor tyrosine-based inhibition motif) and an ITIM-like motif, which are characteristic for the inhibitory function of Siglec-G (Fig. 4).

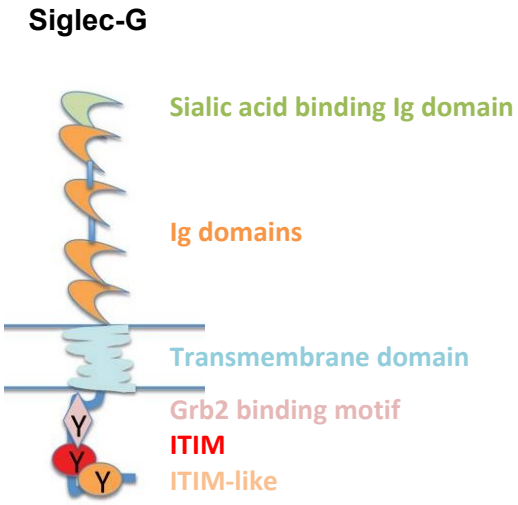

Fig. 4: Structure of Siglec-G. The N-terminal extracellular part consists of an amino-terminal V-set immunoglobulin domain, which is able to bind sialic acids. This is connected to a set of immunoglobulin domains, which length varies among different Siglecs. The intracellular domain on the C-terminus contains several signal motifs: the Grb2 binding motif, the ITIM and the ITIM-like motif. ITIM, immunoreceptor tyrosine-based inhibition motif. Modified from (Pillai et al, 2012).

It is speculated that Siglec-G exerts its inhibitory function via the association with the BCR, whereby Siglec-G inhibits the intracellular signal transduction (Hoffmann et al, 2007). Siglec-G is a direct target of the transcription factor Pax5 (Schebesta et al, 2007) and is mainly

expressed on B cells (Hoffmann et al, 2007; Su et al, 2004). The expression of Siglec-G by B cells was confirmed by the generation of a Siglec-G knockin mouse (Siglec-G was substituted by GFP), however Siglec-G is additionally also expressed to a lower extent on myeloid ($CD11b^+$), dendritic cells ($CD11c^+$) and T cells ($CD3^+$) (Ding et al, 2007). Within the B cell population Siglec-G is expressed from the pro-B cell stadium on all naïve B cells, as has been demonstrated by RT-PCR. Of note, the expression of Siglec-G is highest on B-1a cells (Ding et al, 2007; Hoffmann et al, 2007).

Beside the expression of Siglec-G on B cells, CD22 represents another Siglec being expressed on B cells (Nitschke et al, 1997). As almost all Siglecs, Siglec-G is a receptor which is able to bind sialic acids as ligands. *In vitro* studies addressing the ligand specificity of Siglec-G have revealed broad ligand specificity towards sialic acids. For example, Siglec-G does not only bind to α-2,6 sialic acids but also to α-2,3 sialic acids (Munday et al, 2001) (Munday *et al.,* 2001). These binding specificities have later been confirmed in primary murine B cells (Duong et al, 2010).

Siglec-G has been cloned as a CD22 homolog and its biological function was elucidated via the generation of a Siglec-G-deficient mouse model. In this regard, Siglec-G has been identified as a previously unknown inhibitory receptor on the B-1a cell subpopulation (Hoffmann et al, 2007). Siglec-G-deficient mice exhibit up to 8-fold higher numbers of peritoneal B-1a cells as well as higher splenic B-1 cells. In contrast, B cell development as well as other B cell populations remained unchanged compared to control mice. Moreover, peritoneal B-1a cells show increased calcium signaling after anti-IgM BCR stimulation, whereas splenic B2 cells do not. Therefore, Siglec-G has been identified as an inhibitory receptor of the BCR-mediated calcium signaling on B-1 cells.

1.4.2. B-1a cell expansion in Siglec-G deficiency

The exact mechanisms leading to inhibition of the BCR signal or to the expansion of the B-1 cell population remain unknown. The phosphorylation of a variety of BCR-associated signal molecules, such as PLCγ, Btk and BLNK have been shown to be unchanged in mice deficient in Siglec-G compared to controls (Hoffmann et al, 2007). Notably, Siglec-G-deficient B-1a cells exhibit increased levels of the transcription factor NFATc1, which is activated in a calcium-dependent manner by the phosphatase calcineurin (Jellusova et al, 2010a). Interestingly, mice

deficient in NFATc1 develop a specific defect in B-1 cell development, whereas B2 cells are intact (Berland & Wortis, 2002; Bhattacharyya et al, 2011). This suggests a possible explanation of the expanded B-1 cell population upon Siglec-G deficiency. Moreover, NFATc1 is speculated to play a protective role in the anti-IgM-induced apoptosis of B cells, which could be related to the prolonged survival of Siglec-G-deficient B-1a cells and has been shown *in vitro* and *in vivo* (Jellusova et al, 2010a). Furthermore, an altered selection of B cells into the B-1a population could be responsible for the increased number of B-1 cells in Siglec-G-deficient mice. B-1a cells stem from the fetal liver and use certain canonical VDJ segments and few N-nucleotide insertions in order to generate the BCRs (Baumgarth et al, 2005). It is suspected that the BCR repertoire in Siglec-G-deficient mice is shifted towards the repertoire of B cells which stems from the adult bone marrow. This is characterized by the BCRs which carry more nucleotide insertions and less typical B-1a cell canonical sequences (Jellusova et al, 2010a). Thus, it seems as if in Siglec-G-deficient mice more B cells get selected from the bone marrow into the B-1 cell compartment which consequently results in an expanded population of B-1 cells. Moreover, the expanded B-1 cell population could be directly associated with the stronger BCR signal in B-1a cells of Siglec-G-deficient mice.

In general, it is well established that the loss of an inhibitory signal molecule leads to an increase in the B-1a cell population. In contrast, loss of an activator of BCR signaling results in decreased B-1a cells (Berland & Wortis, 2002).

Brenner *et al*., have crossed Siglec-G-deficient mice onto IgM hypomorphic mice, which show a normal B-1a cell population. Notably, IgM hypomorphic mice are associated with lower BCR levels and hence B-1a cells are nearly absent. Thus it can be speculated that crossing Siglec-G-deficient mice with hypomorphic mice results in the compensation of a low expression of IgM by the missing inhibitory regulation via Siglec-G consequently resulting in normal B-1a cell numbers as seen in wild type mice (Brenner et al, 2011). Taken together, this study provides evidence for an important role of the BCR signal in the context of B-1a cell size.

Importantly, Siglec-G deficiency results in markedly elevated natural IgM antibodies (Hoffmann et al, 2007), which is most likely a consequence of a higher number of IgM secreting cells in the bone marrow and spleen upon Siglec-G deficiency (Choi et al, 2012; Hoffmann et al, 2007).

1.4.3. Regulation of the BCR-mediated signaling in B-1 cells by Siglec-G

As mentioned above, Siglec-G is an inhibitory receptor which exerts its main function as inhibiting BCR-mediated signaling. The stimulation of the BCR by binding of an antigen leads to the phosphorylation of tyrosine residues (Y) of the ITIM of Siglec-G. Consequently, this leads to the recruitment and the activation of the phosphatase SHP-1 (Src homology 2 domain containing protein tyrosine phosphatase 1), which mediates the inhibition of Ca^{2+} signaling from the BCR (Jellusova & Nitschke, 2011) (see Fig. 5).

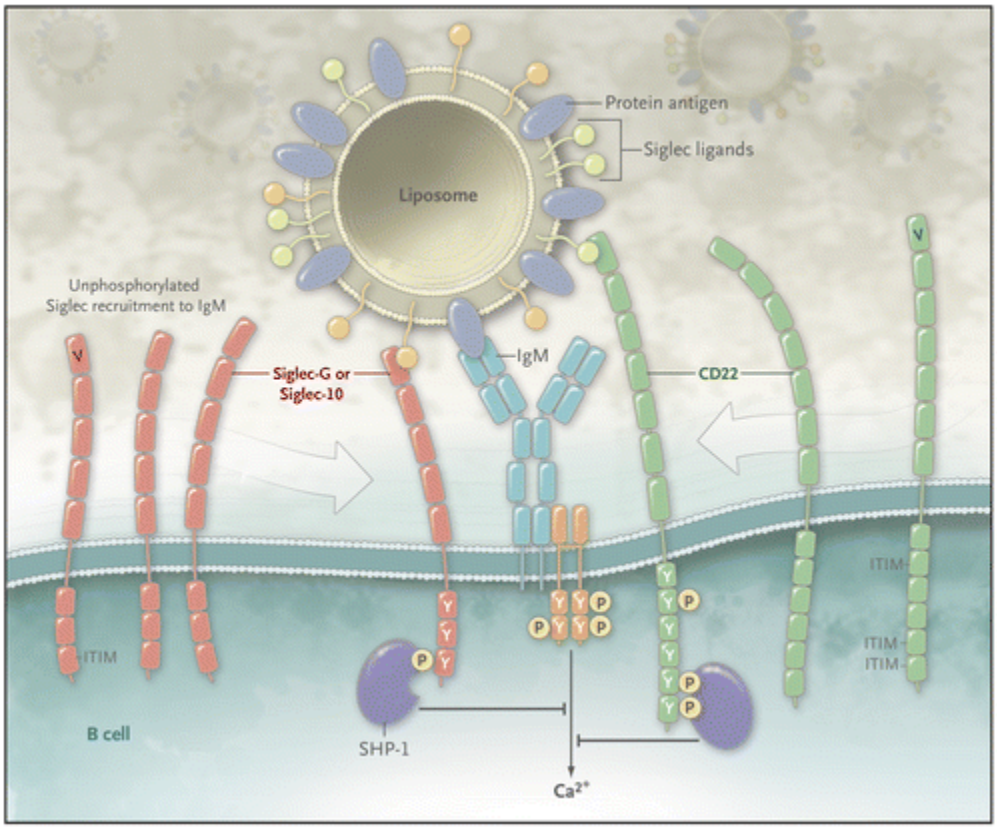

Fig. 5: Inhibition of BCR-mediated signaling in B-1 cells by Siglec-G. Siglec-G is an inhibitory receptor found to be expressed primarily on B cells. After binding of an antigen to the BCR, tyrosine residues (Y) of the ITIM of Siglec-G become phosphorylated. Consequently, the phosphatase SHP-1 gets recruited which then suppresses signaling from the BCR (Figure adapted from (Nitschke, 2013)). Siglec-G, sialic acid–binding immunoglobulin-like lectin G; BCR, B cell receptor; ITIM, immunoreceptor tyrosine-based inhibition motif; SHP-1, Src homology 2 domain containing protein tyrosine phosphatase 1.

1.4.4. Functions of Siglec-G in other cells

Besides its role as a negative regulator of BCR signaling, Siglec-G confers also inhibitory effects on TLR signaling. Siglec-G-deficient B cells that have been stimulated with LPS, which is a prototypic TLR-4 ligand, R848 (TLR-7 ligand) or CpG (TLR-9 ligand) show increased proliferation (Jellusova et al, 2010b), suggesting inhibition of the respective signal pathways by Siglec-G. The mechanisms by which Siglec-G negatively influences TLR signaling remain unclear.

Furthermore, two studies have shown that Siglec-G on dendritic cells plays a role in the discrimination between PAMPs and DAMPS. The first study showed an interaction of Siglec-G with CD24 in an acetaminophen-induced liver necrosis model and the selective inhibition of DAMPs (Chen et al, 2009). In this regard, CD24 binds to DAMPs, such as HMGB1, HSP70 and HSP90. However the mechanisms of CD24 in signal transduction is not known. In more detail this study has shown that CD24 forms a cis-complex with Siglec-G due to its high glycosylation status and is consequently present as a HMGB1/CD24/Siglec-G complex and consequently inhibiting the cytokine response by dendritic cells. Thus, mice deficient for either Siglec-G or CD24 in the acetaminophen (AAP)-induced liver necrosis model display increased levels of pro-inflammatory cytokines, severe liver necrosis and increased mortality. In contrast, *in vitro* experiments of bone marrow-derived DC stimulated with TLR ligands, such as LPS of poly I:C as well as *in vivo* experiments of *i.p.* injection of these stimuli into mice did not show any differences in the inflammatory cytokine production in the supernatants or the serum between the groups tested (Chen et al, 2009). Another study performed in a poly-bacterial sepsis model revealed that sialidases produced by bacteria are able to disrupt the Siglec-G/CD24 interaction, thereby increasing inflammation in the respective tissue – independent of bacterial load and induction of TLRs (Chen et al, 2011). Consequently, CD24- and Siglec-G-deficient mice exhibit higher mortality rates due to sepsis.

Collectively, Siglec-G has been suggested to play a role in the selective inhibition of DAMPs via the dampening of tissue injury after infection. However it is not known which cells confer infection-mediated tissue injury.

1.5. Aims of this thesis

Chronic inflammation evolves from persistence of inflammatory responses to endogenous sterile triggers, such as OxLDL, dying cells, and their metabolic byproducts, which consequently propagate pathologies such as vascular and hepatic inflammation (Tall & Yvan-Charvet, 2015). There it is of importance to identify mechanisms that enhance the immune system's capacity to prevent endogenously-triggered inflammation. In this regard, B cells have been shown to be emerging players in the chronic inflammation of metabolic diseases, such as obesity, diabetes, and atherosclerosis (Harmon et al, 2016; Perry et al, 2012; Tsiantoulas et al, 2014; Winer et al, 2014; Zouggari et al, 2013). B cells are a heterogeneous population including different subsets with different properties. For example, B2 cells have been shown to promote atherosclerotic lesion formation (Ait-Oufella et al, 2010; Kyaw et al, 2010), while selective transfer of B-1 cells protects mice from atherosclerosis (Kyaw et al, 2011; Rosenfeld et al, 2015). Of note, B-1 cells are the main producers of natural IgM antibodies and it is suggested that these promote the neutralization and clearance of self-antigens (Tsiantoulas et al, 2012). Therefore, it is of importance to selectively regulate individual B cell subsets for appropriate responses to inflammatory triggers. Moreover, the role of B-1 cells in atherosclerosis has only been studied in immune-compromised animals, and their role in animals that do not lack major compartments of the immune system remains elusive. In this regard, Siglec-G is of particular interest as it acts as a negative regulator of the B-1a cell population size, presumably via inhibiting BCR dependent signaling (Ding et al, 2007; Hoffmann et al, 2007). It has been previously shown that mice deficient in Siglec-G exhibit a nearly 10-fold expansion of B-1a cells along with a robust increase in total serum IgM (Ding et al, 2007; Hoffmann et al, 2007). Moreover, my thesis laboratory also found that Siglec-G deficiency results in an expansion of IgM with specificity for OSE, which represent prototypic metabolic byproducts present on OxLDL, dying cells, and circulating microparticles (Chang et al, 1999; Chang et al, 2004; Chou et al, 2009; Jellusova et al, 2010a; Tsiantoulas et al, 2015). Of note, excessive accumulation of OSE has been suggested to be a key driver for inflammatory reactions in metabolic diseases, such as atherosclerosis, NASH, and diabetes (Horie et al, 1997; Miller et al, 2011; Walenbergh et al, 2013). Thus, targeting Siglec-G may have beneficial therapeutic effects in chronic inflammation. In essence, the role of Siglec-G and the consequences of Siglec-G deficiency in chronic inflammation and specifically in atherosclerosis are entirely unknown. Thus, I aimed to investigate the role of Siglec-G in sterile chronic inflammation *in vivo*.

2. RESULTS

Sialic acid-binding immunoglobulin-like lectin G promotes atherosclerosis and liver inflammation by suppressing the protective functions of B-1 cells

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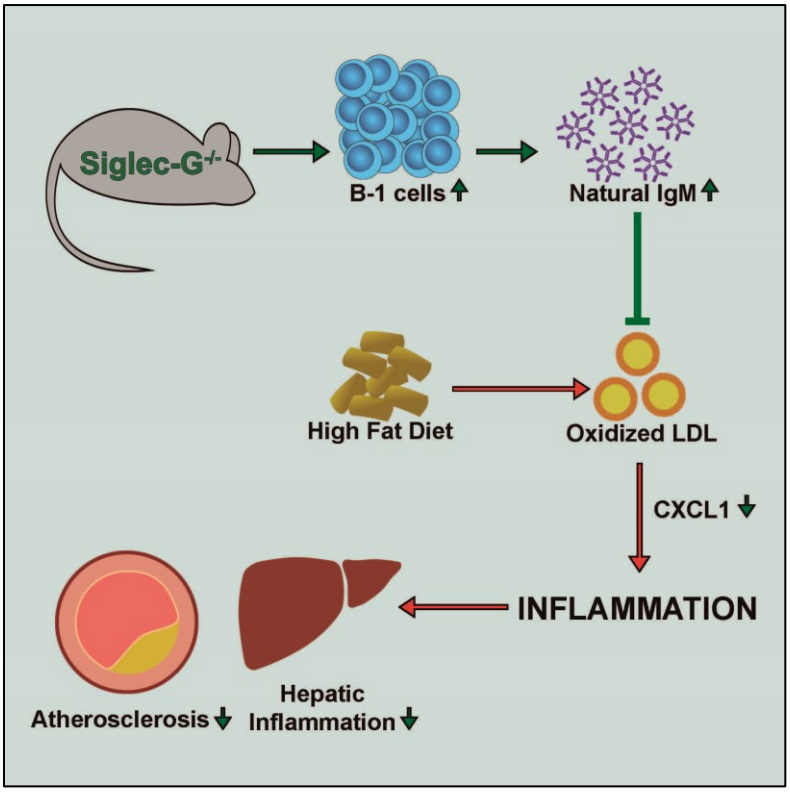
Running Title: Siglec-G deficiency protects from atherosclerosis

Graphical Abstract



Highlights

- Siglec-G deficiency reduces atherosclerosis and hepatic inflammation in Ldlr ko mice
- B cells secreting natural IgM mediate the protective effects of Siglec-G deficiency
- Siglec-G deficiency protects from inflammation induced by oxidized LDL
- CXCL1 is a common mediator of inflammation that is decreased by Siglec-G deficiency

In Brief

Gruber *et al.* demonstrate that Siglec-G deficiency protects from oxidized LDL-induced inflammation through the expansion of B-1 cells secreting natural IgM antibodies leading to reduced atherosclerosis and hepatic inflammation. CXCL1 represents a common pro-inflammatory factor that is reduced as a result of Siglec-G deficiency.

Summary

Atherosclerosis is initiated and sustained by hypercholesterolemia, which results in the generation of oxidized LDL (OxLDL) and other metabolic byproducts that trigger inflammation. Specific immune responses have been shown to modulate the inflammatory response during atherogenesis. The sialic acid-binding immunoglobulin-like lectin G (Siglec-G) is a negative regulator of the functions of several immune cells, including myeloid cells and B-1 cells. Here we show that deficiency of Siglec-G in atherosclerosis-prone mice inhibits plaque formation and diet-induced hepatic inflammation. We further demonstrate that selective deficiency of Siglec-G in B cells alone is sufficient to mediate these effects. Levels of B-1 cell-derived natural IgM with specificity for OxLDL were significantly increased in the plasma and peritoneal cavity of Siglec-G-deficient mice. Consistent with the neutralizing functions of OxLDL-specific IgM, Siglec-G-deficient mice were protected from OxLDL-induced sterile inflammation. Thus, Siglec-G promotes atherosclerosis and hepatic inflammation by suppressing protective anti-inflammatory effector functions of B cells.

Introduction

Atherosclerosis is a lipid-driven chronic disease of the artery wall and the underlying cause of heart attacks and strokes, which accounts for the majority of mortalities and morbidities in the world (Libby et al., 2011). It is characterized by chronic inflammatory responses to endogenous sterile triggers, such as OxLDL, dying cells, and their metabolic byproducts that trigger tissue inflammation if not efficiently cleared (Hotamisligil, 2006; Tabas, 2010). Persistence of this inflammatory response or its impaired resolution paves the way for chronic inflammatory responses, which have been shown to propagate associated pathologies such as vascular and hepatic inflammation (Tall and Yvan-Charvet, 2015). Thus, there is growing interest in identifying mechanisms that enhance the immune system's capacity to prevent endogenously triggered inflammation and/or promote its resolution.

B cells, which can be subdivided into B-1 and B2 cells, are emerging players in the chronic inflammation of metabolic diseases, such as obesity, diabetes, and atherosclerosis (Perry et al., 2012; Tsiantoulas et al., 2014; Winer et al., 2014, Zouggari et al., 2013). B2 cells, which include follicular (FO) B cells and marginal zone (MZ) B cells, have been shown to promote atherosclerotic lesion formation in murine models of atherosclerosis via mechanisms that are largely unclear (Ait-Oufella et al., 2010; Kyaw et al., 2010). On the other hand selective transfer of B-1 cells, which can be further divided into B-1a and B-1b cells, protects mice from atherosclerosis (Kyaw et al., 2011; Rosenfeld et al., 2015). One of the main functions of B-1 cells is the production of natural IgM (NAb), which are pre-existing germline encoded antibodies that arise without any conventional T cell help and comprise approximately 80% of IgM antibodies in unchallenged mice (Baumgarth et al., 2005). B-1a cells seem to exhibit their atheroprotective effects via the secretion of NAb (Tsiantoulas et al., 2014). Indeed, atherosclerosis-prone soluble IgM-deficient mice develop accelerated atherosclerosis, though the exact mechanism by which NAb protect is not entirely clear (Lewis et al., 2009). We and others have suggested that NAb promote the neutralization and clearance of self-antigens, such as dying cells and oxidized lipids (Tsiantoulas et al., 2012). These studies indicate the importance of selective regulation of individual B cell subsets for appropriate responses to inflammatory triggers. Moreover, the role of B-1 cells in atherosclerosis has only been studied in immune-compromised animals, and their role in animals that do not lack major compartments of the immune system remains elusive. In this regard, the sialic-acid binding immunoglobulin-like lectin G (Siglec-G) is of particular interest as it acts as negative regulator of the B-1a cell population size, presumably via inhibiting B cell receptor dependent signaling (Ding et al., 2007;

Hoffmann et al., 2007). We and others have previously shown that mice deficient in Siglec-G exhibit a nearly 10-fold expansion of B-1a cells along with a robust increase in total serum IgM (Ding et al., 2007; Hoffmann et al., 2007). Moreover, we also found that Siglec-G deficiency results in an expansion of IgM with specificity for oxidation-specific epitopes (OSE), which represent prototypic metabolic byproducts present on OxLDL, dying cells, and circulating microparticles (Chang et al., 1999; Chang et al., 2004; Chou et al., 2009; Jellusova et al., 2010; Tsiantoulas et al., 2015). As excessive accumulation of OSE has been suggested to be a key driver for inflammatory reactions in metabolic diseases, such as atherosclerosis, NASH, and diabetes (Horie et al., 1997; Miller et al., 2011; Walenbergh et al., 2013), targeting Siglec-G may have beneficial therapeutic effects in chronic inflammation.

The expansion of B-1a cells has also been associated with increased autoimmunity (Chan et al., 1997; Ishida et al., 2006; Pao et al., 2007), which could accelerate atherosclerosis (Ma et al., 2008; Roman and Salmon, 2007). Siglec-G deficiency has been shown to result in an earlier onset of autoimmune disease in the MRL/lpr lupus mouse model and leads to mild autoimmunity in aging mice with an over-activation of adaptive T and B cells (Bokers et al., 2014; Muller et al., 2015). In addition, Siglec-G has also been found to be expressed in and influence responses of myeloid cells. For example, Siglec-G has been shown to be upregulated by RNA viruses and to inhibit retinoic acid-inducible gene 1 (RIG-I) mediated IFN-β secretion by macrophages and dendritic cells. In line with this, VSV-infected Siglec-G-deficient mice were found to display increased IFN-β production and decreased viral load compared to control mice (Chen et al., 2013). Moreover, dendritic cells of Siglec-G-deficient mice have been found to exhibit increased pro-inflammatory cytokine secretion in response to multiple danger-associated molecular patterns (DAMPs) (e.g. HSP70, HSP90, and HMGB1). A detrimental role of Siglec-G deficiency is further supported by the findings that Siglec-G-deficient mice exhibit increased mortality in models of acetaminophen-induced liver necrosis (Chen et al., 2009) and cecal ligation and puncture-induced sepsis (Chen et al., 2011).

Thus, all studies so far indicate that Siglec-G functions as negative regulator of inflammation, and Siglec-G deficiency may actually propagate inflammatory responses. Because pro-inflammatory cytokine production is a hallmark of metabolic inflammation, the role of Siglec-G and the consequences of Siglec-G deficiency in chronic inflammation and specifically in atherosclerosis are entirely unknown.

Here, we investigated the role of Siglec-G in sterile chronic inflammation *in vivo*. We demonstrate that total as well as B cell-specific Siglec-G deficiency reduces atherosclerotic lesion formation as well as hepatic inflammation in hypercholesterolemic *Ldlr*$^{-/-}$ mice. Moreover, we show that Siglec-G-deficient mice are protected form OxLDL-induced inflammation *in vivo*.

Results

Siglec-G deficiency in cholesterol-fed Ldlr$^{-/-}$ *mice increases B-1a cells and natural IgM antibodies.*

Siglec-G deficiency has been previously shown to result in an expansion of OSE-specific NAb, which are hypothesized to possess robust anti-inflammatory properties, particularly against products of increased oxidative stress (Jellusova et al., 2010). In agreement with previous data, non-atherosclerotic *Siglec-G*$^{-/-}$ mice have - compared to *Siglec-G*$^{+/+}$ control mice - significantly increased numbers of B-1a cells in the spleen, increased numbers of CD138^{+} plasmablasts (B220^{+}) and plasma cells (B220^{lo}) in the spleen and bone marrow, and increased levels of OSE-specific IgM antibodies in the plasma (Figure S1A-G). In order to investigate the effect of Siglec-G deficiency in atherosclerosis, *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice as well as control *Ldlr*$^{-/-}$ mice were fed an atherogenic diet for 8 weeks. Siglec-G deficiency did not affect body weight, or plasma total cholesterol (TC) or triglycerides (TG) (Table 1). Moreover, there was no significant difference in frequencies of total or Ly6C^{hi} and Ly6C^{lo} monocytes in the peripheral blood of these mice (Figure S2E-F). Frequencies of peripheral blood B220^{+} B cells were also not different between the two groups (Figure S2D).

Consistent with the previously described effect of Siglec-G deficiency on B cells, numbers of splenic CD43^{+} B-1 cells were increased in *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice (Figure 1A and 1B). Numbers of CD3^{+} T cells, CD23^{+} FO B cells, and MZB cells were not different between the groups (Table 1). Notably, the numbers of the recently identified pro-atherogenic subset of innate response activator (IRA) B cells in the spleen were significantly increased in *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice compared to *Ldlr*$^{-/-}$ mice (Hilgendorf et al., 2014) (Figure S2A). Moreover, compared to *Ldlr*$^{-/-}$ mice, *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice exhibited a significantly increased frequency of B-1a cells in the peritoneal cavity (Figure 1C and 1D), while the frequencies of B-1b or B2 cells were not significantly different between the groups (Figure S2B and S2C).

We then assessed both total and OSE-specific IgM and IgG antibody levels in plasma at baseline and after 8 weeks of atherogenic diet. Levels of IgM antibodies were significantly increased in *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice at both time points (Figure 1E), while levels of total IgG antibodies were not different (Figure 1F). Moreover, IgM titers to MDA-LDL as well as to CuOx-LDL were also higher in *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice at baseline and even further increased in response to atherogenic diet feeding compared to controls (Figure 1G and 1H). Importantly, expression of the antigen-specific IgM as a ratio of total IgM, revealed a preferential expansion of IgM to MDA-LDL and CuOx-LDL, while the relative levels of IgM to the atherosclerosis-irrelevant antigen α-1,3-dextran were decreased (Figure 1I). On the other hand, IgG titers to these antigens were elevated in *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice only at baseline, but the differences disappeared after 8 weeks of atherogenic diet (Figure S2G and S2H).

Thus, even in the presence of extreme hypercholesterolemia, Siglec-G deficiency results in an expansion of B-1a cells and a concomitant increase of total and OSE-specific IgM.

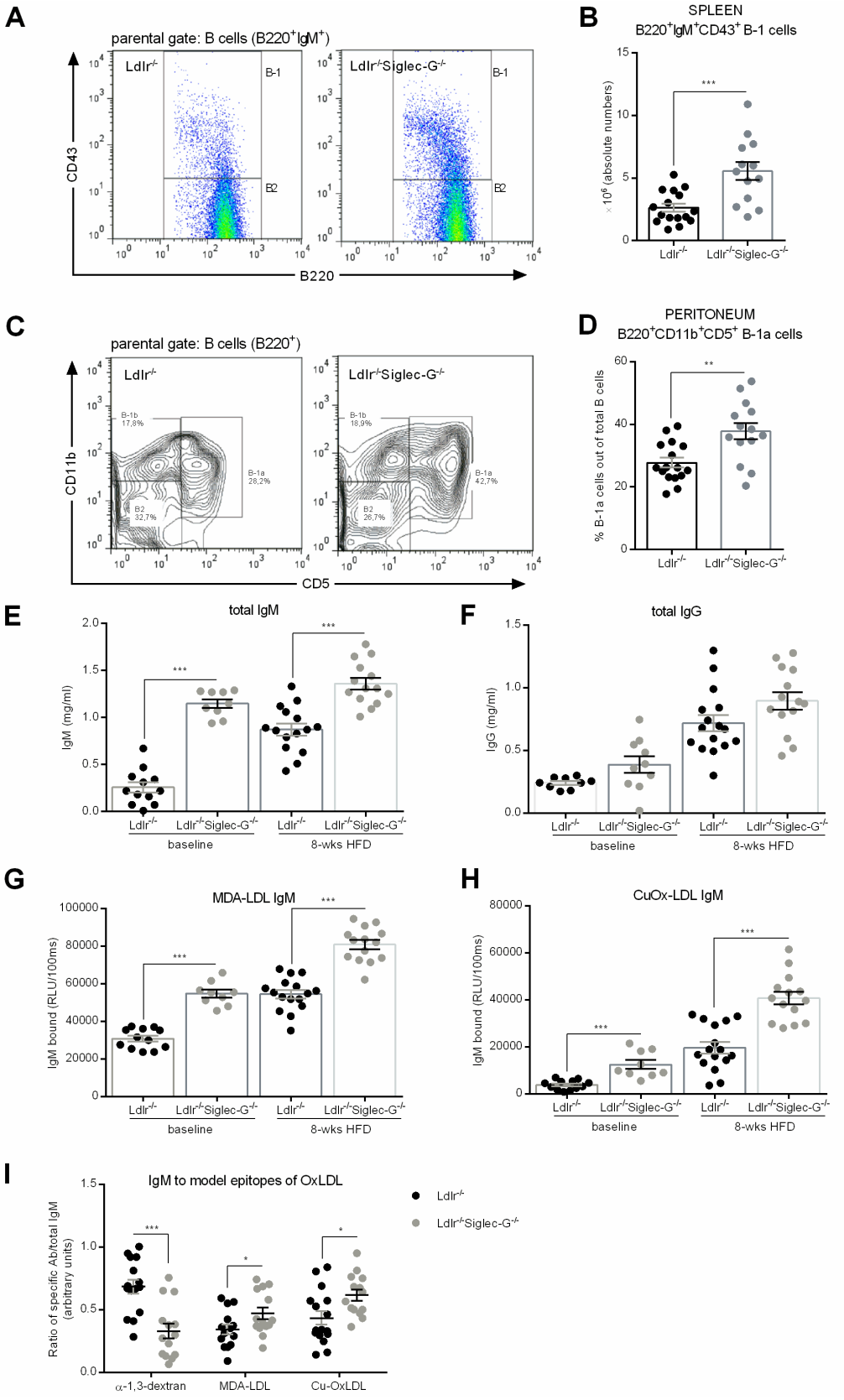


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Figure 1. Siglec-G deficiency in cholesterol-fed *Ldlr*$^{-/-}$ mice increases B-1a cell-derived IgM antibodies. (A) Representative flow cytometry plots for splenic B220^{+}CD43^{-} B2 cells and B220^{+}CD43^{+} B-1 cell subpopulations. The parental gate was set on B220^{+}IgM^{+} B cells. (B) Absolute numbers of splenic B220^{+}IgM^{+}CD43^{+} B-1 cells. (C) Representative flow cytometry plots for B cell subpopulations of the peritoneal cavity. Parental gate was set on B220^{+} B cells. (D) Relative numbers of peritoneal B220^{+}CD5^{+}CD11b^{+} B-1a cells out of total B cells. (E-F) Quantification of total IgM (E) and total IgG (F) antibodies in plasma. Samples were diluted between 1:30,000 and 1:70,000 and measured in triplicates. (G-H) Titers of MDA-LDL IgM (G) and CuOx-LDL IgM (H) in plasma were determined by ELISA at baseline and after 8 weeks of atherogenic diet. Samples were diluted between 1:100 and 1:500 and antibody binding was measured in triplicates. Data are expressed as relative light units (RLU) per 100ms. (I) RLU of IgM antibodies to MDA-LDL and CuOx-LDL as well as α-1,3-dextran (1:100) were normalized to total IgM concentrations and expressed as ratio of specific IgM per total IgM (arbitrary units). Shown are data of *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice after 8 weeks of atherogenic diet. Symbols represent individual mice. Horizontal bars represent the mean of each group and errors bars represent SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. See also Figures S1 and S2.

Total and B cell-selective Siglec-G deficiency protects from atherosclerosis.

We then quantified the extent of atherosclerosis in the entire aorta of both groups of mice by *en face* analyses, which revealed a 50% reduced lesion formation as a result of Siglec-G deficiency (Figure 2A). Decreased atherosclerosis was also found in the innominate arteries of *Ldlr*$^{-/-}$ *Siglec-G*$^{-/-}$ mice compared to *Ldlr*$^{-/-}$ mice (Figure 2B). Moreover, while cross-sectional analyses of the aortic origin did not reveal differences in lesion size between the two groups (Figure 2C), lesions of *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice were clearly less complex with smaller necrotic areas and reduced macrophage content (Figure 2D and 2E). Lesional collagen content was not significantly different between the two groups (Figure 2F), while deposition of IgM was significantly increased in lesions of *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice (Figure 2G). Thus, Siglec-G deficiency results in a profound reduction of lesion size and complexity.

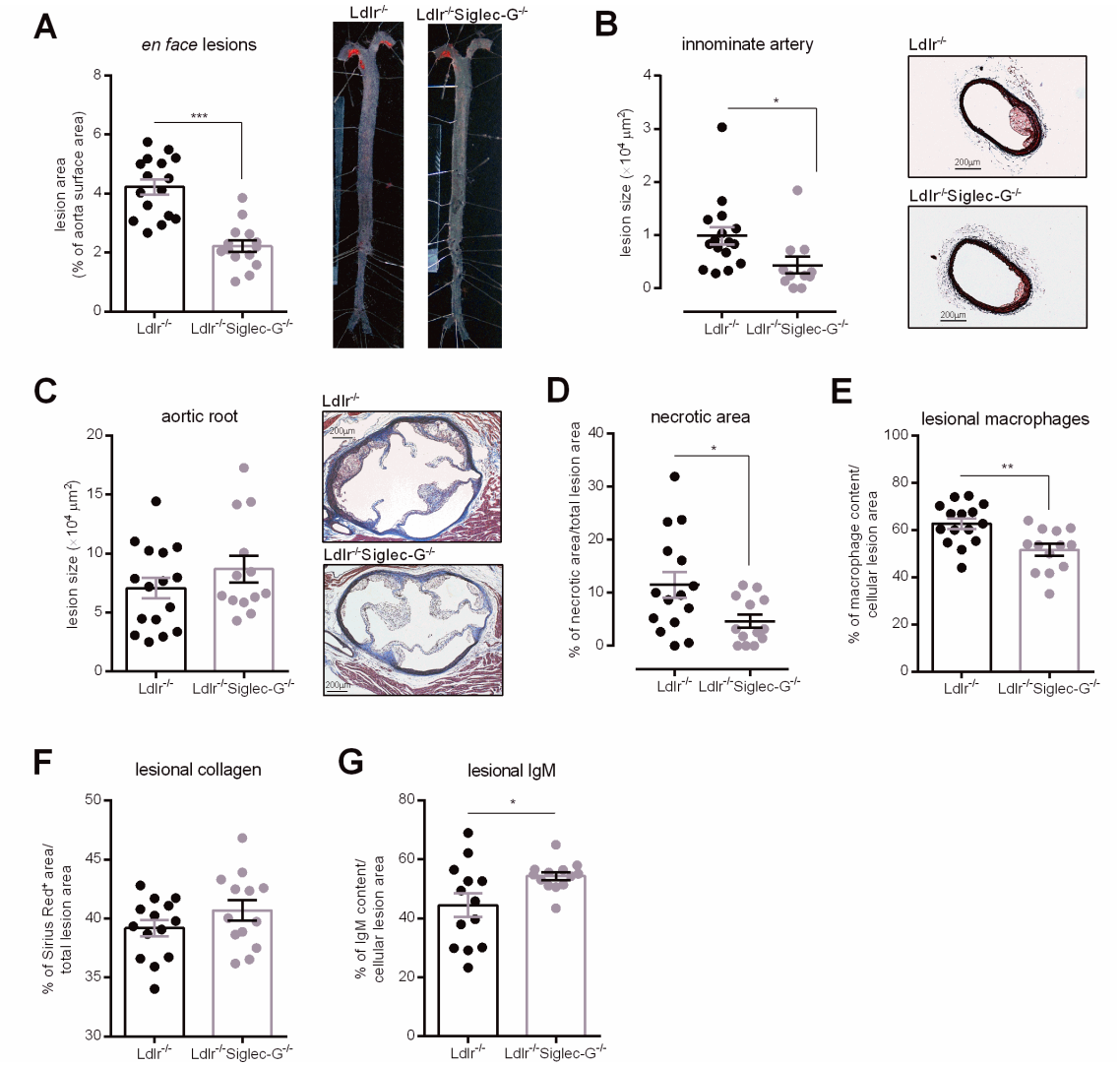

Figure 2. Siglec-G deficiency decreases atherosclerosis in cholesterol-fed *Ldlr$^{-/-}$Siglec-G$^{-/-}$* mice. (A) Quantitative analysis of atherosclerosis in the aorta. Data are expressed as percentage of Sudan IV stained area of the entire aorta. Representative pictures are shown on the right. (B) Quantitative analysis of atherosclerotic lesions in cross-sections of innominate arteries. Values represent the average µm^2 of 4 sections throughout the innominate artery (300 µm). Images show representative Masson's trichrome stains. Original magnification 50×, scale bar 200 µm. (C) Quantification of atherosclerotic lesion size in cross-sections at the aortic origin. Values represent the average µm^2 of 9 sections throughout the entire aortic origin (400 µm). Images show representative Masson's trichrome stains. Original magnification 50×, scale bar 200 µm. (D) Quantification of necrotic areas in cross-sections at the aortic origin. Values represent the percentages of necrotic areas of one section at 200 µm depth. (E-G) Quantification of macrophage, collagen, and IgM content in cross-sections at the aortic origin. (E) Values represent the percentage of mac-3$^+$ area per cellular lesion area of one section per mouse at 200 µm depth. (F) Values represent the percentage of Sirius Red$^+$ area per total lesion area of one section per mouse at 200 µm depth. (G) Values represent the percentage of IgM$^+$ area per cellular lesion area of one section per mouse at 180 µm depth. Shown are data of *Ldlr$^{-/-}$* and *Ldlr$^{-/-}$Siglec-G$^{-/-}$* mice after 8 weeks of atherogenic diet. Symbols represent individual

mice. Horizontal bars represent the mean of each group and errors bars represent SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. See also Table 1.

To identify whether the observed protective effects of Siglec-G deficiency are mainly conferred by B cells, we generated bone marrow chimeric *Ldlr*$^{-/-}$ mice with a selective Siglec-G deficiency on B cells using a previously established method (Fillatreau et al., 2002; Sage et al., 2012). *Ldlr*$^{-/-}$ mice were lethally irradiated and reconstituted with 1) bone marrow from *Siglec-G*$^{-/-}$ mice or *Siglec-G*$^{+/+}$ littermate controls to assess the role of Siglec-G deficiency in hematopoietic cells in general, and 2) a mixture of 80% bone marrow from B cell-deficient *μMT* mice and 20% from either *Siglec-G*$^{-/-}$ mice or *Siglec-G*$^{+/+}$ littermate controls to assess Siglec-G deficiency specifically in B cells. After a 4 week recovery period, mice were fed an atherogenic diet for 10 weeks. Successful engraftment of the respective bone marrow was confirmed by PCR of DNA isolated from bone marrow of recipients and of sorted splenic B and non-B cells to demonstrate selective Siglec-G deficiency (Figure S3A and S3B). In addition, the lack of Siglec-G expression on splenic B cells was confirmed by flow cytometry (Figure S3C).

Body weights, TC and TG levels in plasma, as well as frequencies of peripheral blood monocytes and B cells were not different between the experimental groups (Table 2 and Figure S4A-C). Splenic B-1 cells as well as peritoneal B-1a cells were significantly increased in recipients of *Siglec-G*$^{-/-}$ bone marrow (whole and mixed bone marrow) compared to recipients of control bone marrow (Figures 3A and S4D; Table 2). Moreover, total plasma IgM levels were significantly increased in mice reconstituted with *Siglec-G*$^{-/-}$ and μMT+*Siglec-G*$^{-/-}$ bone marrow compared to their controls (Figure 3B), while total plasma IgG levels were not different (Figure S4E). IgM – but not IgG - levels to MDA-LDL and CuOx-LDL were also robustly and preferentially increased in recipient mice of *Siglec-G*$^{-/-}$ bone marrow (Figures 3C, 3D and S4F, S4G, S4H). Importantly, total and B cell-selective deficiency of Siglec-G in the hematopoetic compartment resulted in a similar reduction of atherosclerotic lesion formation (Figure 3E) and a decreased lesional complexity with smaller necrotic areas and fewer lesional macrophages in the aortic root (Figure 3F-3H). This indicates that Siglec-G deficiency on B cells is primarily responsible for the protective effects in atherogenesis.

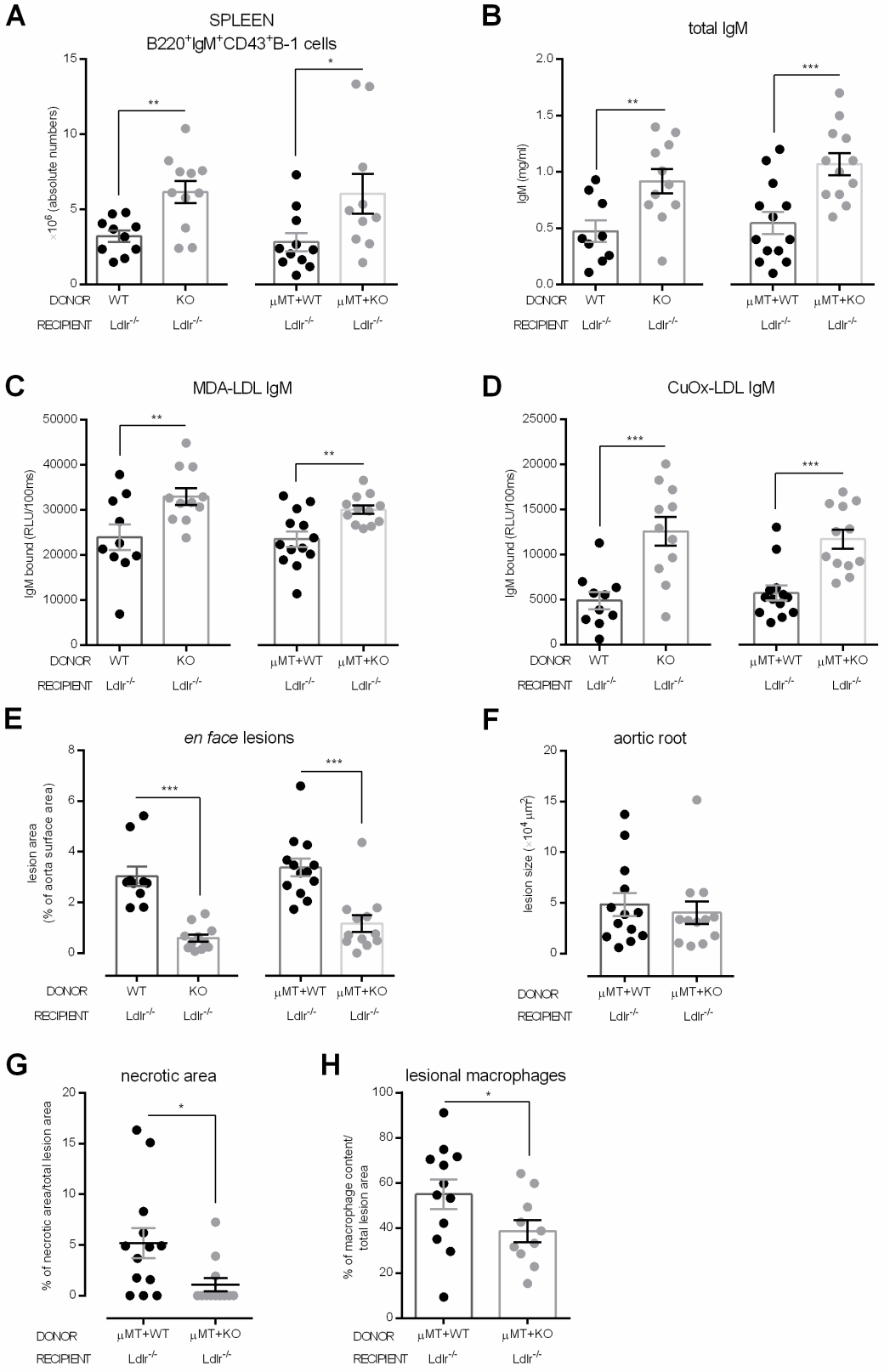

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Figure 3. Selective Siglec-G deficiency in B cells reduces the development of atherosclerosis. (A) Absolute numbers of splenic $IgM^+B220^+CD43^+$ B-1 cells. (B) Quantification of total IgM in plasma. Samples were diluted 1:60,000 and measured in triplicates. (C-D) Binding of IgM antibodies to (C) MDA-LDL and (D) CuOx-LDL was determined by ELISA. Plasma samples were diluted between 1:100 and 1:500 and antibody binding was measured in triplicates. Data are expressed as RLU/100ms. (E) Quantitative analysis of atherosclerosis *(en face)* in the entire aorta. Data are expressed as percentage of Sudan IV stained area of the entire aorta. (F) Quantification of atherosclerotic lesion size in cross-sections at the aortic origin. Values represent the average μm^2 of 9 sections throughout the entire aortic origin (400 µm). (G) Quantification of necrotic areas in cross-sections at the aortic origin. Values represent percentages of necrotic areas of one section at 200 µm. (H) Quantification of the macrophage content in cross-sections at the aortic origin. Values represent percentages of mac-3^+ area per total lesion area of one section per mouse at 200 µm depth. Shown are data of *Ldlr*$^{-/-}$ mice reconstituted with *Siglec-G*$^{+/+}$ [WT] vs. *Siglec-G*$^{-/-}$ [KO] and µMT+*Siglec-G*$^{+/+}$ [WT] vs. µMT+*Siglec-G*$^{-/-}$ [KO] bone marrow after 10 weeks of atherogenic diet. Symbols represent individual mice. Horizontal bars represent the mean of each group and errors bars represent SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. See also Figures S3, S4 and Table 2.

Siglec-G deficiency in mice protects from hepatic and systemic inflammation.

We have recently demonstrated that *Ldlr*$^{-/-}$ mice develop hepatic inflammation when fed an atherogenic diet (Bieghs et al., 2012b). Therefore, we investigated the effect of Siglec-G deficiency on hepatic inflammation in these mice. Atherogenic diet feeding resulted in a robust steatosis of the liver of all experimental mice. We found no significant differences in hepatic cholesterol, hepatic triglycerides, and hepatic free fatty acids between the experimental groups Consistent with only minimal liver damage in this model, ALT levels were only moderately elevated, but also not different between the two groups (Table 1 and 2). In contrast, immunohistochemical analyses of liver sections revealed a significantly reduced infiltration of $Ly6G^+$ neutrophils and mac-1^+ macrophages in the livers of *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice compared to *Ldlr*$^{-/-}$ mice (Figure 4A and 4B). A similar significant reduction in neutrophil and macrophage infiltration was found in the livers of *Ldlr*$^{-/-}$ mice that were reconstituted with either *Siglec-G*$^{-/-}$ or µMT+*Siglec-G*$^{-/-}$ bone marrow (Figure S5A and S5B). To further define the effects on hepatic inflammation between *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice, we analyzed the expression of adhesion molecules, pro-inflammatory cytokines, and chemokines *in the liver of these mice.* In line with the reduced cell infiltration, expression of intercellular *adhesion* molecule (*Icam*) and vascular cell *adhesion* protein (*Vcam*), tumor necrosis factor-alpha (*Tnf-α*) and interleukin-18 (*Il-18*), C-C motif ligand 5 (*Ccl5*), C-X-C motif ligand 1 (*Cxcl1*) and C-X-C motif ligand 2 (*Cxcl2*) was significantly reduced in the livers of *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice compared to *Ldlr*$^{-/-}$ mice (Figure 4C). A similar though less pronounced decrease of inflammatory gene expression was also observed

in the livers of mice lacking Siglec-G in B cells only (Figure S5C). Thus, Siglec-G deficiency and Siglec-G deficiency on B cells reduces hepatic inflammation, suggesting an important role in the development of steatohepatitis.

To assess systemic markers of inflammation in these mice we quantified hepatic mRNA expression and the circulating levels of serum amyloid A (SAA), which in mice represents an acute phase protein that is induced during chronic inflammation (Uhlar and Whitehead, 1999). Expression of *Saa1* was significantly reduced in the livers of *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice compared to *Ldlr*$^{-/-}$ mice (Figure 4C). Moreover, while plasma SAA levels increased after eight weeks of atherogenic diet in *Ldlr*$^{-/-}$, this was not the case for *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice (Figure 4D). SAA levels in *Ldlr*$^{-/-}$ mice that were reconstituted with µMT+*Siglec-G*$^{-/-}$ or µMT+*WT* bone marrow after 10 weeks of atherogenic diet were lower in both groups consistent with a more moderate degree of inflammation, but still showed a tendency towards reduced levels as a result of Siglec-G deficiency (Figure S5D). To better characterize the inflammatory responses in these mice, we quantified the plasma levels of cytokines (TNF-α, IL-6, and IL-18) and chemokines (CCL2, CXCL1, and CXCL2) that have been previously identified to contribute to different pathogenic responses in atherosclerosis and hepatic inflammation (Ait-Oufella et al, 2011; Weber & Noels, 2011). Of all cytokines and chemokines tested, plasma levels of CXCL1 were significantly reduced both in *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice (Figure 4E) and *Ldlr*$^{-/-}$ mice lacking Siglec-G in B cells only compared to their respective controls (Figure S5E). Notably, Cxcl1 has been shown to be prominently induced in macrophages and endothelial cells stimulated with OxLDL, and mediates the recruitment of neutrophils and monoctyes (Berliner et al., 1990; Stewart et al., 2010).

Thus, atherogenic diet is associated with increased inflammation in *Ldlr*$^{-/-}$ mice, and this effect is suppressed in mice lacking Siglec-G. Collectively, these data point to a strong anti-inflammatory effect of Siglec-G deficiency in diet-induced hepatic and systemic inflammation.

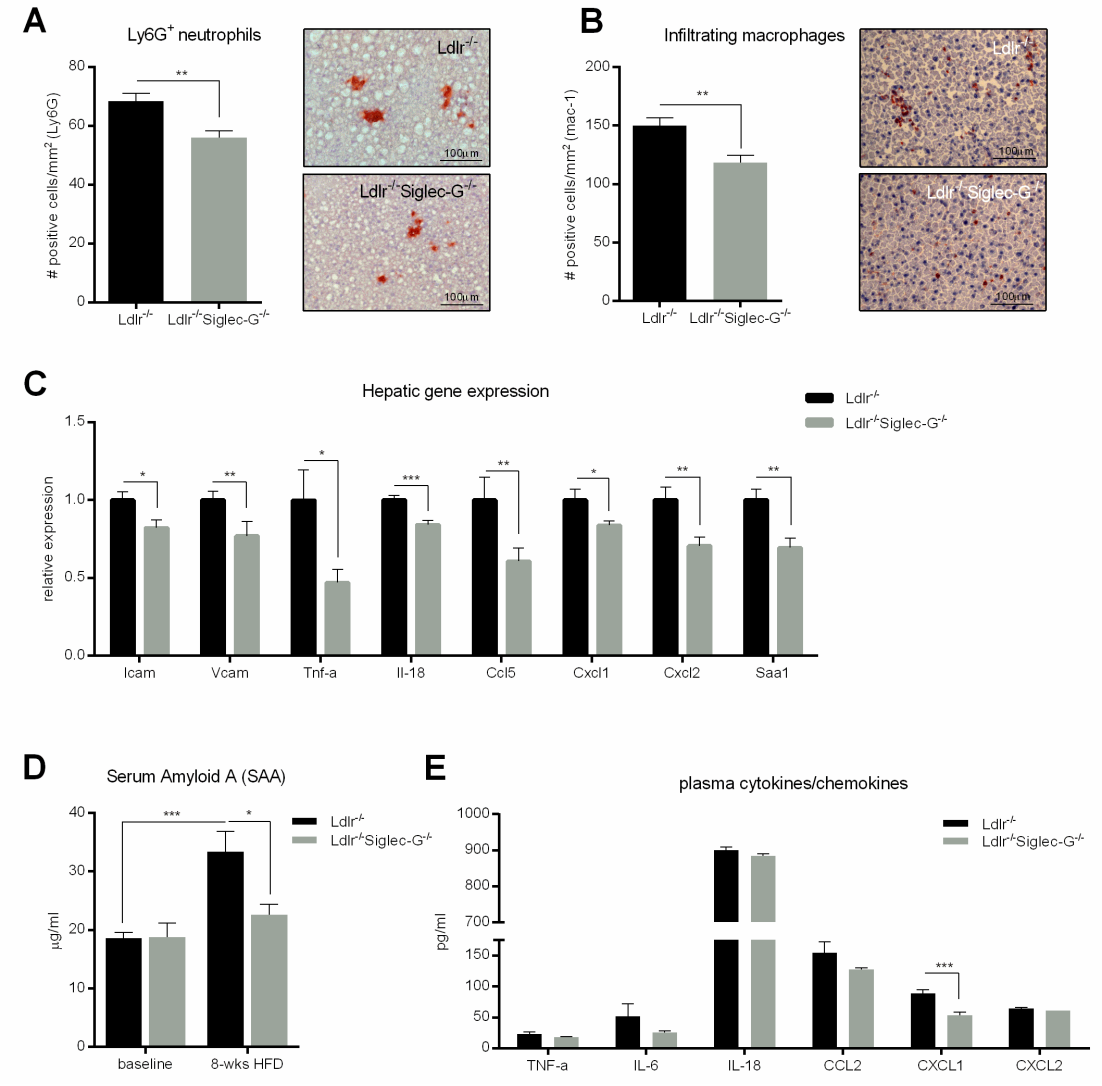

Figure 4. Siglec-G deficiency protects from atherogenic diet-induced hepatic and systemic inflammation. (A, B) Quantification of infiltrating neutrophils (A) and macrophages (B) in liver sections of *Ldlr*$^{-/-}$ vs. *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice after 8 weeks of atherogenic diet. Sections were stained with anti-Ly6G and anti-mac-1 antibody. Positively stained cells were counted and expressed as number of positive cells per mm^2. Representative pictures (original magnification 200×, scale bar 100 µm) are shown on the right. (C) Relative gene expression of *Icam, Vcam, Tnf-α, Il-18, Ccl5, Cxcl1, Cxcl2* and *Saa1* mRNA in livers of *Ldlr*$^{-/-}$ vs. *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice after 8 weeks of atherogenic diet. The expression data of individual genes was normalized to the house-keeping gene S12 and expressed relative to the expression in *Ldlr*$^{-/-}$ control mice. (D) Quantification of SAA levels in plasma of *Ldlr*$^{-/-}$ vs. *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice at baseline and after 8 weeks of atherogenic diet. Samples were diluted 1:300 and measured in triplicates. (E) Quantification of TNF-α, IL-6, IL-18, CCL2, CXCL1 and CXCL2 in plasma of *Ldlr*$^{-/-}$ vs. *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice after 8 weeks of atherogenic diet by multiplex assay. Samples were diluted 1:2. Data represent mean ± SEM of 9-13 mice per group. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. See also Figure S5.

Siglec-G deficiency protects from OxLDL-induced sterile peritonitis.

Because we have previously shown that OSE-specific NAb inhibit pro-inflammatory effects of epitopes of OxLDL (Imai et al., 2008; Tsiantoulas et al., 2015), which is considered a major driver of vascular and hepatic inflammation (Miller et al., 2011; Walenbergh et al., 2013), we evaluated the effect of Siglec-G deficiency in an OxLDL-induced sterile inflammation model *in vivo*. For this, we first established a sterile peritonitis model that allowed us to monitor the recruitment of neutrophils and monocytes in the peritoneal lavage fluid (PLF) of wild type C57BL/6 mice 2-, 6-, and 12 hours after they had received intraperitoneal (*i.p.*) injection of OxLDL. Compared to baseline, mice injected with OxLDL exhibited a marked recruitment of Ly-6G^{+} neutrophils into the peritoneal cavity already after 2 hours, which remained significantly different but gradually declined after 6 and 12 hours (Figures 5A, S6 and S7A). Similarly, peritoneal recruitment of Ly-6C^{+}CD11b^{int} inflammatory monocytes was also induced following *i.p.* injection of OxLDL, but only peaked 6 hours after the injection, consistent with the delayed recruitment of monocytes during inflammatory processes (Figures 5B and S7B).

To investigate the anti-inflammatory effect of natural IgM associated with Siglec-G deficiency, we compared the dynamics of inflammatory cell recruitment during OxLDL-induced peritonitis in *Siglec-G*$^{+/+}$ and *Siglec-G*$^{-/-}$ mice, respectively. Consistent with the dramatic increase of IgM antibodies in the plasma of *Siglec-G*$^{-/-}$ mice, we observed >3-fold higher levels of total and >9-fold higher levels of OxLDL-specific IgM in the PLF of *Siglec-G*$^{-/-}$ mice at baseline (Figure 5C and 5D). At baseline the numbers of peritoneal macrophages, which represent the cellular population that is primarily involved in responding to OxLDL, were not significantly different between *Siglec-G*$^{+/+}$ and *Siglec-G*$^{-/-}$ mice (Figure S7C). *Siglec-G*$^{-/-}$ mice showed a significantly reduced recruitment of neutrophils 2 hours after *i.p.* injection of OxLDL compared to *Siglec-G*$^{+/+}$ mice, while after 6 hours no differences between the two groups were observed (Figure 5E). Consistent with this, OxLDL-induced secretion of CXCL1 and CXCL2 was nearly absent in the peritoneal cavity of *Siglec-G*$^{-/-}$ mice at this time point (Figure 5G and 5H). Six hours after *i.p.* injection of OxLDL, the recruitment of Ly-6C^{+}CD11b^{int} inflammatory monocytes appeared lower in *Siglec-G*$^{-/-}$ mice (Figure 5F). In contrast, Siglec-G deficiency had no effect on neutrophil recruitment in response to injection of thioglycollate (another sterile trigger), indicating that Siglec-G-deficient mice do not have defective inflammatory responses in general and demonstrating specificity for inflammatory responses triggered by OSE (Figure S7D). In summary, Siglec-G deficiency results in a significant inhibition of the inflammatory response to OxLDL *in vivo*. The markedly reduced infiltration of neutrophils in *Siglec-G*$^{-/-}$ mice in response to

i.p. injection of OxLDL indicates direct neutralization of its pro-inflammatory moieties and more efficient clearance mediated by OSE-specific IgM.

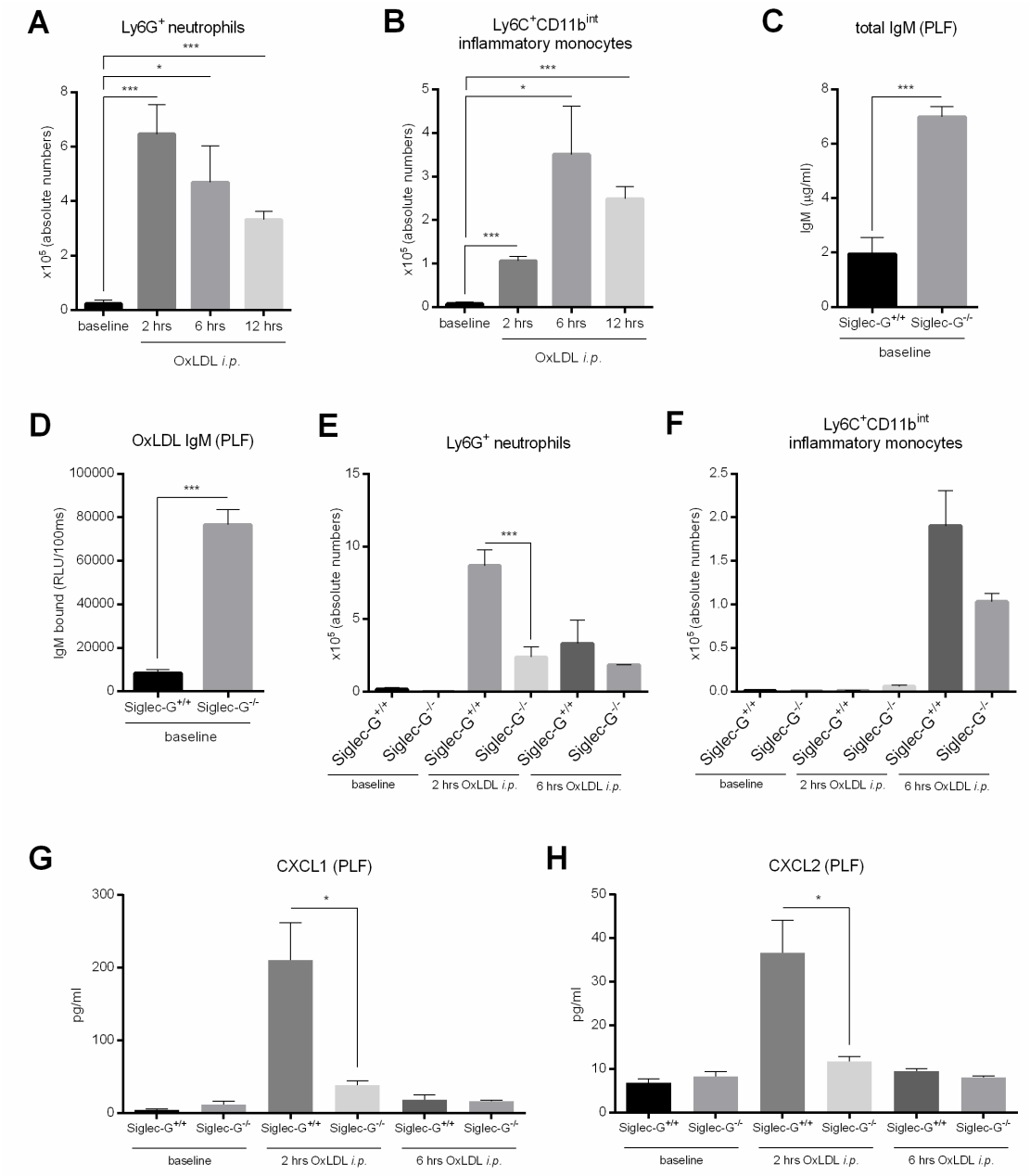

Figure 5. OxLDL-induced sterile peritonitis is suppressed by Siglec-G deficiency. (A-B) Quantification of absolute numbers of Ly6G^{+} neutrophils (A) and Ly6C^{hi} inflammatory monocytes (B) in the PLF of C57BL/6 mice at baseline, as well as 2, 6, and 12 hours after OxLDL injections. (C) Quantification of total IgM in the PLF of *Siglec-G*$^{+/+}$ and *Siglec-G*$^{-/-}$ mice. Samples were diluted 1:20 and measured in triplicates. (D) Binding of IgM to CuOx-LDL in the

PLF of *Siglec-G*$^{+/+}$ and *Siglec-G*$^{-/-}$ mice was determined by ELISA. Samples were undiluted and antibody binding was measured in triplicates. Data are expressed as relative RLU/100 ms. (E-F) Quantification of absolute numbers of Ly6G^{+} neutrophils (E) and Ly6C^{hi} monocytes (F) in the PLF of *Siglec-G*$^{+/+}$ and *Siglec-G*$^{-/-}$ mice at baseline, 2 and 6 hours after *i.p.* injection of OxLDL. (G-H) Quantification of CXCL1 (G) and CXCL2 (H) in the PLF of *Siglec-G*$^{+/+}$ and *Siglec-G*$^{-/-}$ mice at indicated time points by ELISA. Samples were undiluted and measured in duplicates. Data represent mean ± SEM of 3-7 mice per group and are a representative example of 3 individual experiments. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. See also Figures S6 and S7.

	Ldlr$^{-/-}$ (*n* = 16)	*Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ (*n* = 14)
Metabolic parameters		
Weights (g)	32.15 ± 0.53	31.2 ± 0.90
TC (mg/dL)	1655.36 ± 70.24	1633.65 ± 88.09
TG (mg/dL)	1257.14 ± 55.37	1284.38 ± 61.18
Atherosclerosis		
en face (% of aorta)	4.22 ± 0.25	2.22 ± 0.20***
innominate (×10^4 mm^2)	0.99 ± 0.17	0.43 ± 0.16*
aortic origin (×10^4 μm^2/section)	7.08 ± 0.88	8.69 ± 1.14
necrotic area (% of total lesion area)	14.8 ± 4.0	4.65 ± 1.21*
mac-3^+ (% of cellular area)	65.83 ± 3.78	51.61 ± 2.53**
Hepatic inflammation		
Liver cholesterol (µg TC/µg protein)	0.19 ± 0.01	0.19 ± 0.02
Liver triglycerides (µg TG/µg protein)	0.68 ± 0.04	0.62 ± 0.06
Liver free fatty acids (µg FFA/µg protein)	0.42 ± 0.02	0.37 ± 0.04
ALT (U/l)	72.9 ± 8.1	80.4 ± 6.6
Mac-1 (# positive cells/mm^2)	150.04 ± 6.74	118.58 ± 6.14**
Ly6G (# positive cells/mm^2)	68.38 ± 2.61	55.91 ± 2.32**
Serum antibody titers		
Total IgM (mg/ml)		
Baseline	0.26 ± 0.05	1.15 ± 0.05***
8-weeks HFD	0.87 ± 0.06	1.36 ± 0.06***
MDA-LDL IgM (RLU/100ms)		
Baseline	30735 ± 1534	54680 ± 2101***
8-weeks HFD	54378 ± 2196	80773 ± 2467***
CuOx-LDL IgM (RLU/100ms)		
Baseline	3767 ± 585	12459 ± 1894***
8-weeks HFD	19549 ± 2443	40649 ± 2698***
Total IgG (mg/ml)		
Baseline	0.24 ± 0.02	0.39 ± 0.07
8-weeks HFD	0.72 ± 0.06	0.89 ± 0.07
MDA-LDL IgG (RLU/100ms)		
Baseline	14879 ± 1855	22392 ± 2383*
8-weeks HFD	20646 ± 1800	23699 ± 1870
CuOx-LDL IgG (RLU/100ms)		
Baseline	1473 ± 91	3220 ± 525**
8-weeks HFD	3267 ± 356	4463 ± 449
Spleen (abs. No × 10^6)		
T cells ($CD3^+$)	34.18 ± 2.30	37.97 ± 3.04
B cells ($B220^+IgM^+$)	33.14 ± 2.44	38.64 ± 4.25
B1 cells ($B220^+IgM^+CD43^+$)	2.63 ± 0.32	5.56 ± 0.73***
MZ B cells ($B220^+CD43^-CD23^-CD21^{hi}$)	0.71 ± 0.11	0.54 ± 0.09
T2/FO B cells ($B220^+CD43^-CD23^+CD21^+$)	26.87 ± 2.26	34.0 ± 3.71
T1 B cells ($B220^+CD43^-CD23^-CD21^{lo}$)	1.96 ± 0.18	2.71 ± 0.36
$B220^+CD43^-CD23^-CD21^+$	1.50 ± 0.15	1.84 ± 0.15
IRA B cells ($CD19^+IgM^{high}CD43^+CD5^+CD138^+CD93^+MHCII^+$)	0.09 ± 0.01	0.18 ± 0.03**
Peritoneal cavity (% viable cells)		
B-1a cells ($B220^+CD11b^+CD5^+$) out of B cells	27.74 ± 1.59	37.78 ± 2.60**
B-1b cells ($B220^+CD11b^+CD5^-$) out of B cells	15.57 ± 1.46	12.39 ± 1.41
B2 cells ($B220^+CD11b^-CD5^-$) out of B cells	35.29 ± 1.84	33.2 ± 1.84
T cells ($B220^-CD5^+$) out of total	8.99 ± 1.10	9.42 ± 0.86

Table 1. Parameters overview of experimental groups - atherosclerosis study. TC, total serum cholesterol; TG, serum triglycerides; ALT, alanine transaminase. Data are shown as mean ± SEM. * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001

***Ldlr*$^{-/-}$** ←	**C57BL/6 (*n* = 10)**	***Siglec-G*$^{-/-}$ (*n* = 11)**	***μMT*+C57BL/6 (*n* = 13)**	***μMT+Siglec-G*$^{-/-}$ (*n* = 13)**
Metabolic parameters				
Weights (g)	25.54 ± 0.63	25.15 ± 0.67	25.72 ± 0.49	25.29 ± 0.73
TC (mg/dL)	758.33 ± 79.47	821.59 ± 66.53	884.67 ± 92.20	925.96 ± 78.29
TG (mg/dL)	595.83 ± 49.43	567.05 ± 48.57	661.46 ± 73.94	585.58 ± 46.60
Atherosclerosis				
en face (% of aorta)	3.04 ± 0.38	0.59 ± 0.15***	3.11 ± 0.24	1.17 ± 0.34***
aortic origin (×10^4 μm^2/section)			4.84 ± 1.14	4.03 ± 1.13
necrotic area (% of total lesion area)			5.20 ± 1.48	1.09 ± 0.66*
mac-3$^+$ (% of total area)			54.96 ± 6.57	38.60 ± 4.95*
Hepatic inflammation				
Liver cholesterol (μg TC/μg protein)	0.22 ± 0.04	0.19 ± 0.03	0.23 ± 0.03	0.23 ± 0.03
Liver triglycerides (μg TG/μg protein)	0.25 ± 0.04	0.21 ± 0.02	0.25 ± 0.03	0.22 ± 0.02
Liver free fatty acids (μg FFA/μg protein)	0.14 ± 0.01	0.12 ± 0.01	0.15 ± 0.01	0.15 ± 0.01
ALT (U/l)	109.1 ± 27.2	58.8 ± 11.5	47.6 ± 4.8	38.6 ± 3.0
Mac-1 (# positive cells/mm^2)	120.69 ± 13.08	86.72 ± 8.95*	100.68 ± 6.33	79.41 ± 5.50*
Ly6G (# positive cells/mm^2)	79.67 ± 5.82	58.58 ± 7.41*	74.40 ± 6.30	48.61 ± 4.04**
Serum antibody titers - 10 weeks HFD				
Total IgM (mg/ml)	0.47 ± 0.10	0.92 ± 0.11**	0.55 ± 0.10	1.07 ± 0.10***
MDA-LDL IgM (RLU/100ms)	23939 ± 2848	32989 ± 1848**	23514 ± 1706	30045 ± 943**
CuOx-LDL IgM (RLU/100ms)	4877 ± 951	12566 ± 1583***	5714 ± 829	11704 ± 1045***
Total IgG (mg/ml)	0.47 ± 0.10	0.89 ± 0.10*	0.61 ± 0.09	0.98 ± 0.27
MDA-LDL IgG (RLU/100ms)	3220 ± 633	6126 ± 1248	4405 ± 701	4012 ± 752
CuOx-LDL IgG (RLU/100ms)	2885 ± 754	3476 ± 769	1827 ± 274	1990 ± 452
Spleen (abs. No × 10^6)				
T cells (CD3$^+$)	41.93 ± 4.76	37.51 ± 3.86	67.76 ± 17.05	42.90 ± 8.13
B cells (B220$^+$IgM$^+$)	66.80 ± 9.44	74.18 ± 9.22	43.85 ± 12.07	47.46 ± 13.18
B1 cells (B220$^+$IgM$^+$CD43$^+$)	3.21 ± 0.38	6.15 ± 0.75**	2.82 ± 0.60	6.04 ± 1.32**
MZ B cells (B220$^+$CD43$^-$CD23$^-$CD21^{hi})	1.98 ± 0.35	1.56 ± 0.26	4.58 ± 1.41	2.94 ± 1.28
T2/FO B cells (B220$^+$CD43$^-$CD23$^+$CD21$^+$)	46.04 ± 5.43	54.25 ± 7.49	23.47 ± 7.63	25.28 ± 8.42
T1 B cells (B220$^+$CD43$^-$CD23$^-$CD21^{lo})	3.42 ± 0.77	3.46 ± 0.35	3.68 ± 2.40	2.24 ± 0.72
B220$^+$CD43$^-$CD23$^-$CD21$^+$	5.99 ± 1.18	5.48 ± 0.67	4.16 ± 1.05	5.38 ± 2.08
Peritoneal cavity (% viable cells)				
B-1a cells (B220$^+$CD11b$^+$CD5$^+$) out of B cells	17.13 ± 1.40	31.85 ± 1.14***	25.18 ± 0.62	46.20 ± 1.07***
B-1b cells (B220$^+$CD11b$^+$CD5$^-$) out of B cells	14.17 ± 1.24	12.47 ± 0.97	19.45 ± 1.29	15.96 ± 0.83*
B2 cells (B220$^+$CD11b$^-$CD5$^-$) out of B cells	64.96 ± 2.32	48.47 ± 2.00***	47.40 ± 1.94	29.88 ± 1.06***
T cells (B220$^-$CD5$^+$) out of total	11.99 ± 1.19	15.07 ± 2.68	18.18 ± 5.04	13.97 ± 2.89

Table 2. Parameters overview of experimental groups - bone marrow transplantation study. TC, total serum cholesterol; TG, serum triglycerides; ALT, alanine transaminase. Data are shown as mean ± SEM. * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.

Discussion

In this study we demonstrate a pathogenic role for Siglec-G in atherosclerosis and hepatic inflammation. We could show that Siglec-G deficiency in atherosclerosis-prone *Ldlr*$^{-/-}$ mice leads to a marked reduction of atherosclerotic lesion burden as well as decreased hepatic inflammation. This effect is specific for Siglec-G deficiency in B cells that results in higher B-1 cell numbers and a robust and preferential increase in OSE-specific IgM antibodies, which neutralize OxLDL-induced inflammation *in vivo*.

Siglec-G is primarily expressed on B cells, with slightly higher expression levels on B-1a cells than on conventional B2 cells (Hoffmann et al., 2007), but it has also been shown to be expressed on dendritic cells and eosinophils (Hutzler et al., 2014; Pfrengle et al., 2013). A suppressive role of Siglec-G in non-B cells has been suggested in drug-induced liver damage, as Siglec-G deficiency results in increased production of pro-inflammatory cytokines, including IL-6, TNF-α, and MCP-1, following stimulation with DAMPs that are released in this setting (Chen et al., 2009). Nevertheless, our data clearly identify an anti-inflammatory effect of Siglec-G deficiency in cholesterol-fed *Ldlr*$^{-/-}$ mice as documented by reduced hepatic expression of adhesion molecules, pro-inflammatory cytokines and chemokines as well as lower CXCL1 levels in plasma. Moreover, our data demonstrate Siglec-G deficiency to be protective in a model of OxLDL-induced peritonitis, while it has been shown to aggravate inflammation in cecal ligation and puncture-induced polybacterial peritonitis. These detrimental effects of Siglec-G deficiency have been shown to be dependent on its interaction with the GPI-anchored co-stimulatory protein CD24 (Chen et al., 2011). CD24 has been shown to recognize DAMPs and to allow Siglec-G to suppress pro-inflammatory signaling via pattern-recognition receptors, which can be disrupted by bacterial sialidases but not by LPS. In our study, Siglec-G deficiency conferred a protective effect against another type of sterile trigger, OxLDL, as Siglec-G-deficient mice exhibited decreased inflammation in an OxLDL-induced peritonitis model. Presumably, the sterile inflammation induced by OxLDL also does not disrupt the interaction of CD24 and Siglec-G, suggesting that Siglec-G deficiency protects via other mechanisms than CD24.
It has also been shown that RNA viruses evade anti-viral defenses by up-regulating Siglec-G in macrophages and dendritic cells, resulting in diminished production of type I interferons that are essential in the host defense against viruses (Chen et al., 2013). As a consequence of this, Siglec-G deficiency results in increased production of type I interferons, which notably have been shown to promote atherosclerosis in several studies (Ait-Oufella et al., 2011). Because we have shown that Siglec-G deficiency protects from atherosclerosis, this pathway seems to be

not relevant in this setting. In line with this, stimulation of peritoneal and bone-marrow derived macrophages with OxLDL does not induce Siglec-G expression (data not shown). Taken together, our data argue against a role of Siglec-G in myeloid cells with respect to atherosclerosis and hepatic inflammation. Moreover, we provide clear evidence that the protective effect of Siglec-G deficiency is mediated by B cells, indicating that the function of Siglec-G on B cell is dominant. In B cells, Siglec-G is expressed in all stages throughout B cell development (Hutzler et al., 2014; Pfrengle et al., 2014). Therefore, its functional role in autoimmunity has been studied, particularly in aging mice (Muller et al., 2015). Old *Siglec-G*$^{-/-}$ mice have been shown to exhibit an increased expression of activation markers on T cells and conventional B2 cells, suggesting a more general over-activation of the adaptive immune system in these settings. However, the effects described in our study are more consistent with the increased production of protective B-1 cell-derived NAb by Siglec-G-deficient mice. In line with previous data, we demonstrate that Siglec-G deficiency also resulted in a dramatic expansion of peritoneal B-1a as well as splenic B-1 cells in atherosclerotic mice. FO B cells and MZB cells remained unchanged in *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice as well as in the bone marrow chimeric mice. Thus, our data support a protective role of B-1 cells in atherosclerosis (Tsiantoulas et al., 2014). Notably, we did observe a robust increase of the recently described IRA B cells in the spleens of atherosclerotic *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice. These cells have been suggested to originate from B-1a cells and have been shown to promote atherosclerotic lesion formation (Hilgendorf *et al.*, 2014). Thus, although our data are in line with a B-1 cell origin of IRA B cells, the protective effects of B-1 cells dominate in atherosclerotic mice lacking Siglec-G. Previous studies demonstrated an atheroprotective effect of adoptive transfer of B-1a cells into splenectomized *Apoe*$^{-/-}$ mice and of B-1b cells into *Rag-1*$^{-/-}$*Apoe*$^{-/-}$ mice (Kyaw et al., 2011b; Rosenfeld et al., 2015). These studies address the role of B-1 cells only in immune-compromised mice on top of existing differences in various immune cell populations, such as lack of splenoctyes or total lymphocytes as a result of splenectomy or Rag-1 deficiency, respectively. However, they do not provide a conclusive answer on the role of B-1 cells in atherosclerosis in an intact host. Our data provide clear and direct genetic evidence that expansion of B-1a cells in intact mice mediates atheroprotection.

One of the major functions of B-1 cells is the secretion of natural IgM antibodies, which have been suggested to mediate the atheroprotective effect of B-1 cells. Kyaw *et al*. showed that accelerated lesion formation in splenectomized *Ldlr*$^{-/-}$ mice, which exhibit diminished peritoneal B-1a cell numbers and reduced serum IgM, is reversed by the adoptive transfer of B-1a cells.

This effect was dependent on the capacity of B-1 a cells to secrete IgM, as adoptive transfer of B-1 cells from secreted *IgM*$^{-/-}$ donors did not have any effect (Kyaw et al., 2011b). The atheroprotective role of NAbs is further supported by the fact that *Ldlr*$^{-/-}$ mice deficient in secreted IgM develop increased atherosclerosis compared to *Ldlr*$^{-/-}$ control mice (Lewis et al., 2009). These data also suggested that increasing IgM titers above and beyond normal plasma levels could confer atheroprotection. Interestingly, hypercholesterolemia raises plasma IgM levels, presumably as part of an endogenous defense mechanism against increased accumulation of metabolic waste products (Khoo et al., 2015). However, this protective response is not sufficient to prevent the formation of atherosclerotic plaques and hepatic inflammation. Deficiency of Siglec-G results in an even greater increase of IgM antibodies despite excessive hypercholesterolemia, and thus Siglec-G may be an attractive target for therapeutic intervention, e.g. by blocking antibodies.

Natural IgM exhibit house-keeping functions by promoting the clearance of dying cells and cellular debris (Avrameas, 1991; Notkins, 2004). These properties of natural IgM are in part mediated by their ability to recognize OSE, which are also present in OxLDL (Miller et al., 2011). We have previously shown that immunization of high-fat diet-fed *Ldlr*$^{-/-}$ mice with heat-killed pneumococci led to the expansion of a natural IgM clone recognizing OxLDL (T15/E06) and significantly decreased atherosclerotic lesion formation and hepatic inflammation (Bieghs et al., 2012b; Binder et al., 2003). These data suggested that anti-OxLDL IgM protect from atherosclerosis and liver inflammation. Indeed, epidemiological studies in humans indicate that high levels of OxLDL-specific IgM are associated with a lower incidence of cardiovascular disease (Tsiantoulas et al., 2014). Of note, Siglec-G deficiency, which results in atheroprotection, leads to a preferential increase of IgM with specificity for different epitopes of OxLDL in plasma.

Several mechanisms exist by which OxLDL-specific IgM exhibit atheroprotective properties, including their ability to inhibit foam cell formation by blocking scavenger receptor-mediated uptake of OxLDL by macrophages (Tsiantoulas et al., 2014). In addition, anti-OxLDL IgM prevent the accumulation of apoptotic cells by promoting their uptake by macrophages (Chou et al., 2009; Ogden et al., 2005). In line with this, we show smaller necrotic areas in lesion of *Ldlr*$^{-/-}$ *Siglec-G*$^{-/-}$ mice. Moreover, anti-OxLDL IgM inhibit inflammation by components of OxLDL *in vitro* (Imai et al., 2008). In agreement with a neutralizing effect of anti-OxLDL IgM, we now show reduced CXCL1 and CXCL2 secretion as well as neutrophil infiltration into the peritoneum *in*

vivo following injection of OxLDL into *Siglec-G*$^{-/-}$ mice, which have >9-fold higher levels of anti-OxLDL IgM in the peritoneal cavity compared to wild type controls. Moreover, plasma levels of CXCL1 were significantly reduced in Siglec-G-deficient atherosclerotic mice. OxLDL has been shown to induce Cxcl1 expression in macrophages via a co-operation of CD36/TLR4/TLR6, and CXCL1 represents a key mediator of leukocyte recruitment in atherosclerosis (Stewart et al., 2010). Moreover, both macrophage CD36 and TLR-4 expression have been shown to promote atherogenesis and hepatic inflammation. Thus, increased natural IgM with specificity for OxLDL have the capacity to inhibit atherosclerotic lesion formation and hepatic inflammation in Siglec-G-deficient mice by neutralizing the pro-inflammatory properties of OxLDL.

In conclusion, we provide evidence that expansion of natural IgM antibodies beyond physiological plasma levels protect from atherosclerosis and hepatic inflammation. Exploiting these mechanisms, e.g. by blocking the inhibitory effect of Siglec-G, may represent a therapeutic approach to enhance and strengthen the endogenous defense mechanisms to protect from cardiovascular disease and associated metabolic disorders, such as non-alcoholic steatohepatitis.

Experimental procedures

An expanded version of experimental procedures can be found in Supplemental Experimental Procedures.

Animals and intervention studies

All experimental protocols were approved by the institutional animal experimentation committee and the Austrian Ministry of Science. All mice were on a C57BL/6 background. *Ldlr*$^{-/-}$ and *μMT* mice were purchased originally from The Jackson Laboratories (Bar Harbor, Maine, USA). The generation of C57BL/6 *Siglec-G*$^{-/-}$ mice has been described elsewhere (Muller et al., 2015). *Siglec-G*$^{-/-}$ mice were further crossed with *Ldlr*$^{-/-}$ mice to obtain *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ and *Ldlr*$^{-/-}$*Siglec-G*$^{+/+}$ mice. For intervention studies, three cohorts of 12 weeks-old male *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice (n = 14) and *Ldlr*$^{-/-}$*Siglec-G*$^{+/+}$ (n = 16) littermate controls were fed an atherogenic diet containing 21% milk fat and 0.2% cholesterol (TD88137, Ssniff Spezialdiäten GmbH, Soest, Germany) for 8 weeks. Bone marrow transplantation studies were performed as previously described (Binder et al., 2003; Fillatreau et al., 2002; Sage et al., 2012) and as described in Supplemental Experimental Procedures.

Analysis of atherosclerotic lesions

The extent of atherosclerosis was assessed in a blinded fashion in *en face* preparations of the entire aorta and in cross-sections through the aortic origin by computer-assisted image analysis as previously described (Binder et al., 2004; Binder et al., 2003; Cardilo-Reis et al., 2012).

Liver histology

The left lobes of livers were harvested and 4 equal pieces were snap frozen in liquid nitrogen for further analyses. Frozen liver sections (7 μm) were stained for the presence of infiltrating macrophages and neutrophils as described previously (Bieghs et al., 2012a). For the presence of neutrophils, sections were stained with mouse anti-Ly6G (1:50; BD Pharmingen). Cell nuclei were counterstained with hematoxylin (Klinipath, Duiven, The Netherlands). Pictures were taken with a Nikon digital camera DMX1200 and ACT-1 v2.63 software (Nikon Corporation, Tokyo, Japan). The number of positive-stained cells was counted in six pictures (200×) per liver per mouse to determine the level of liver inflammation.

Flow cytometry

Peritoneal exudate cells (PEC), splenocytes, bone marrow cells, and blood leukocytes were harvested as previously described (Cardilo-Reis et al., 2012) and stained as indicated in Supplemental Experimental Procedures. Stained cell populations were analyzed by multiparameter flow cytometry using a BD FACSCalibur (BD Bioscience, Franklin Lakes, New Jersey, USA) or BD FACS Fortessa, respectively. Dead cells and doublets were excluded by forward- and side-scatter and data were analyzed using the FlowJo v10 data analysis software (Tree Star Inc., Oregon Corporation, Ashland, USA). Criteria used to identify the various cell populations are provided in Table 1.

Plasma antibody and protein analyses

Total IgM levels were measured by a chemiluminescent-based sandwich ELISA as described previously (Chou et al., 2009). Total IgG levels were measured in plasma using a commercially available ELISA (Mouse IgG total ELISA Ready-SET-Go!, eBioscience). Cu^{2+}-oxidized LDL (CuOx-LDL) and MDA-LDL were prepared as described previously (Chou et al., 2009). α-1,3-dextran was a gift of John F. Kearney (University of Alabama at Birmingham, Alabama, USA). Antigen-specific antibody titers were measured by chemiluminescent ELISA as previously described (Binder et al, 2003). OxLDL-specific IgM per total IgM ratios were calculated based on the measurements of specific IgM at non-saturating dilutions (expressed as RLU/100ms) and total IgM quantities of each individual sample. Data are expressed as arbitrary units of these ratios. SAA levels were measured in plasma diluted 1:300 using a commercially available ELISA for mouse SAA (Tridelta Development Ltd.).

Measurement of chemokines/cytokines in plasma and peritoneal lavage fluid

A panel of chemokines and cytokines (IL-6, TNF-α, CCXL1, CXCL2, CCL2 and IL-18) was measured in mouse plasma (1:2 dilution) with ProcartaPlex® Multiplex Immunoassays (eBioscience) according to the manufacturer's protocol. Analysis was performed with the xMAP® technology by Luminex (Austin, TX, USA). Levels of CXCL1 and CXCL2 in the PLF were determined by using mouse CXCL1/KC DuoSet ELISA and Mouse CXCL2/MIP-2 DuoSet ELISA, respectively according to the manufacturer's protocol (R&D systems, Minneapolis, MN, USA).

Murine sterile peritonitis

Siglec-G$^{-/-}$ mice and wild type littermate controls (8–12 weeks of age) were injected *i.p.* with sterile CuOx-LDL (tested for endotoxin levels by chromogenic Limulus amoebocyte assay; < 0.05 ng LPS/mg protein) suspended in 600 µl of sterile PBS-BSA (25 µg/gram body weight) or an comparable volume of sterile thioglycollate (Lipid MAPS) (25 µl/g body weight). At selected time points, mice were sacrificed, and PEC were collected by lavaging the peritoneum with 6 ml of sterile PBS+1% BSA. PEC were pelleted for 10 min at 300 g, and processed for flow cytometry, as described above. Cell-free lavage fluid was stored in aliquots and further processed for ELISA measurements.

Statistical analysis

Data were analyzed using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA). Normal distribution of data was assessed, and statistical analyses were performed by unpaired Student *t*-test to determine statistical significance between the groups. Data points, which were more than 2xSTDEV of the mean, were excluded as statistical outliers. Exclusion of these values did not change the significance of the results. Data are presented as the mean ± standard error of mean (SEM) and considered significant at $P \leq .05$ ($^{*}P \leq .05$, $^{**}P \leq .01$, and $^{***}P \leq .001$, respectively).

Author contributions

S.G. designed and performed experiments, analyzed and interpreted data and wrote the manuscript. T.H. and D.T performed experiments, analyzed and interpreted data and critically revised the manuscript. M.O.K and L.G. performed experiments. J.L.W. provided intellectual input and critically revised the manuscript. Z.M., R.S., L.N. provided technical and material support and critically revised the manuscript. C.J.B. conceived, designed, and supervised the study, drafted and critically revised the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplemental information

Sialic acid-binding immunoglobulin-like lectin G promotes atherosclerosis and liver inflammation by suppressing the protective functions of B-1 cells

Sabrina Gruber, Tim Hendrikx, Dimitrios Tsiantoulas, Maria Ozsvar-Kozma, Laura Göderle, Ziad Mallat, Joseph L. Witztum, Ronit Shiri-Sverdlov, Lars Nitschke and Christoph J. Binder

Supplemental Information Inventory

Supplemental Figures

Figure S1.

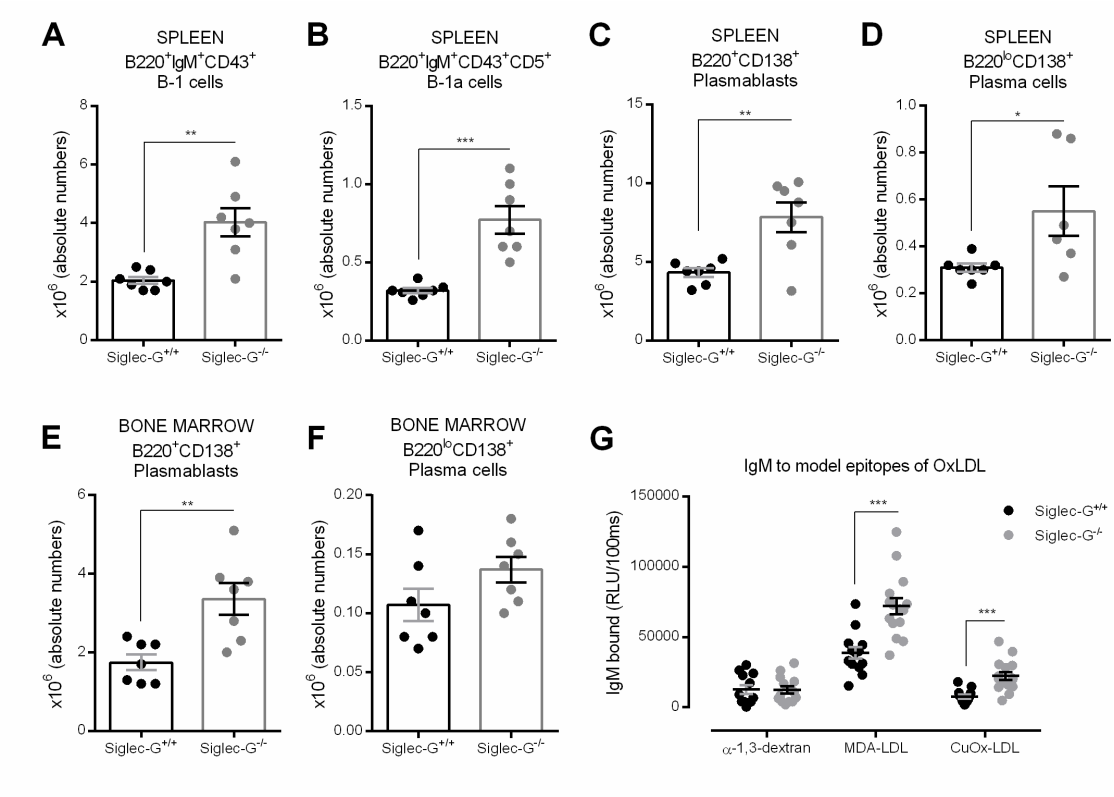


Figure S2.

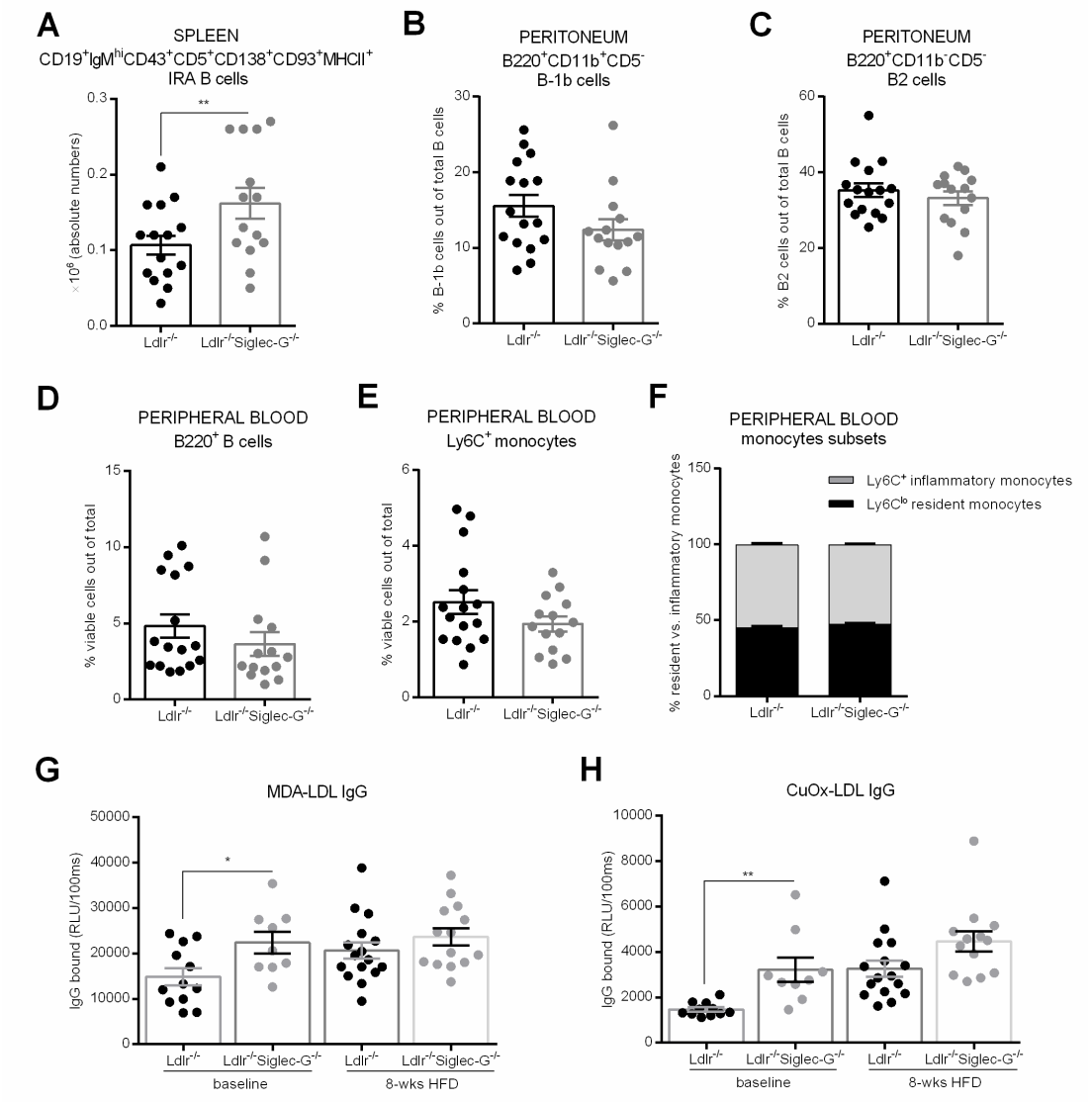

A
SPLEEN
CD19+IgMhiCD43+CD5+CD138+CD93+MHCII+
IRA B cells
x 10^6 (absolute numbers)
B
PERITONEUM
B220+CD11b+CD5-
B-1b cells
% B-1b cells out of total B cells
C
PERITONEUM
B220+CD11b-CD5-
B2 cells
% B2 cells out of total B cells
D
PERIPHERAL BLOOD
B220+ B cells
% viable cells out of total
E
PERIPHERAL BLOOD
Ly6C+ monocytes
% viable cells out of total
F
PERIPHERAL BLOOD
monocytes subsets
Ly6C+ inflammatory monocytes
Ly6Clo resident monocytes
% resident vs. inflammatory monocytes
G
MDA-LDL IgG
IgG bound (RLU/100ms)
H
CuOx-LDL IgG
IgG bound (RLU/100ms)
Ldlr-/-
Ldlr-/-Siglec-G-/-
baseline
8-wks HFD

Figure S3.

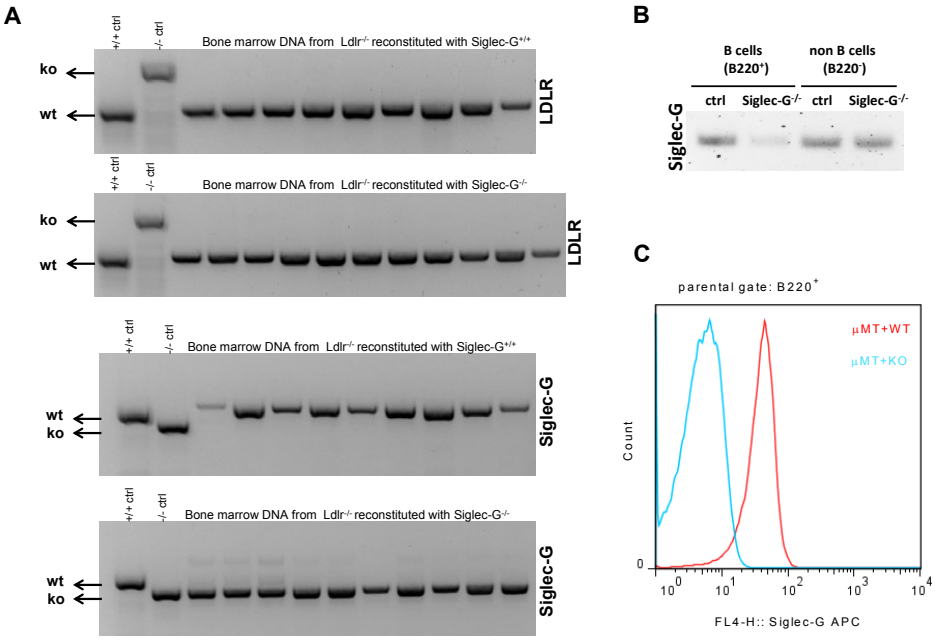

A
+/+ ctrl
-/- ctrl
Bone marrow DNA from Ldlr-/- reconstituted with Siglec-G+/+
ko
wt
LDLR
Bone marrow DNA from Ldlr-/- reconstituted with Siglec-G-/-
Bone marrow DNA from Ldlr-/- reconstituted with Siglec-G+/+
Siglec-G
Bone marrow DNA from Ldlr-/- reconstituted with Siglec-G-/-
B
B cells (B220+)
non B cells (B220-)
ctrl
Siglec-G-/-
Siglec-G
C
parental gate: B220+
µMT+WT
µMT+KO
Count
FL4-H:: Siglec-G APC

Figure S4.

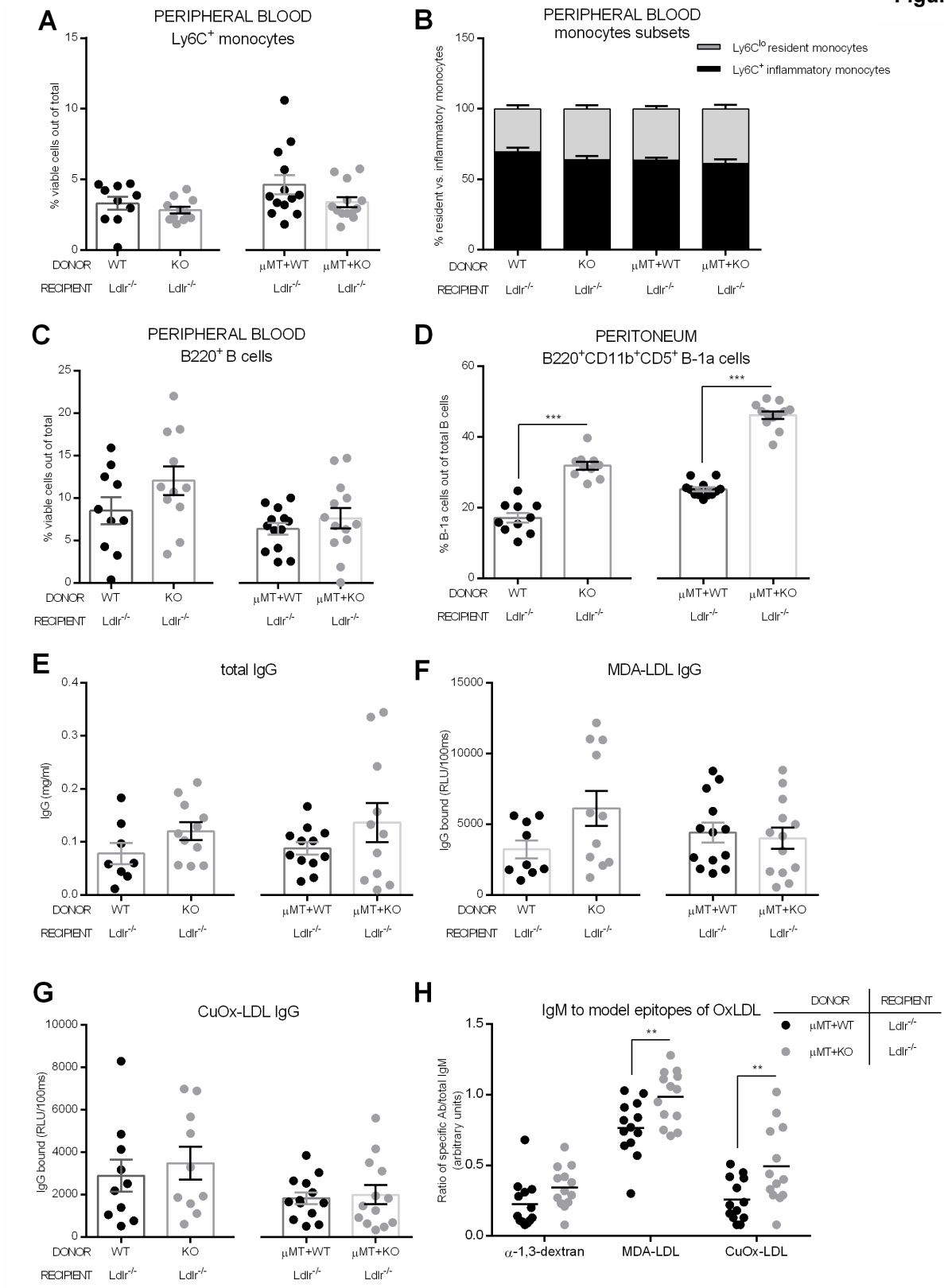

A
PERIPHERAL BLOOD
Ly6C+ monocytes
% viable cells out of total
DONOR WT KO µMT+WT µMT+KO
RECIPIENT Ldlr-/- Ldlr-/- Ldlr-/- Ldlr-/-
B
PERIPHERAL BLOOD
monocytes subsets
Ly6Clo resident monocytes
Ly6C+ inflammatory monocytes
% resident vs. inflammatory monocytes
C
PERIPHERAL BLOOD
B220+ B cells
D
PERITONEUM
B220+CD11b+CD5+ B-1a cells
% B-1a cells out of total B cells
***
E
total IgG
IgG (mg/ml)
F
MDA-LDL IgG
IgG bound (RLU/100ms)
G
CuOx-LDL IgG
H
IgM to model epitopes of OxLDL
DONOR RECIPIENT
µMT+WT Ldlr-/-
µMT+KO Ldlr-/-
Ratio of specific Ab/total IgM (arbitrary units)
**
α-1,3-dextran
MDA-LDL
CuOx-LDL

Figure S5.

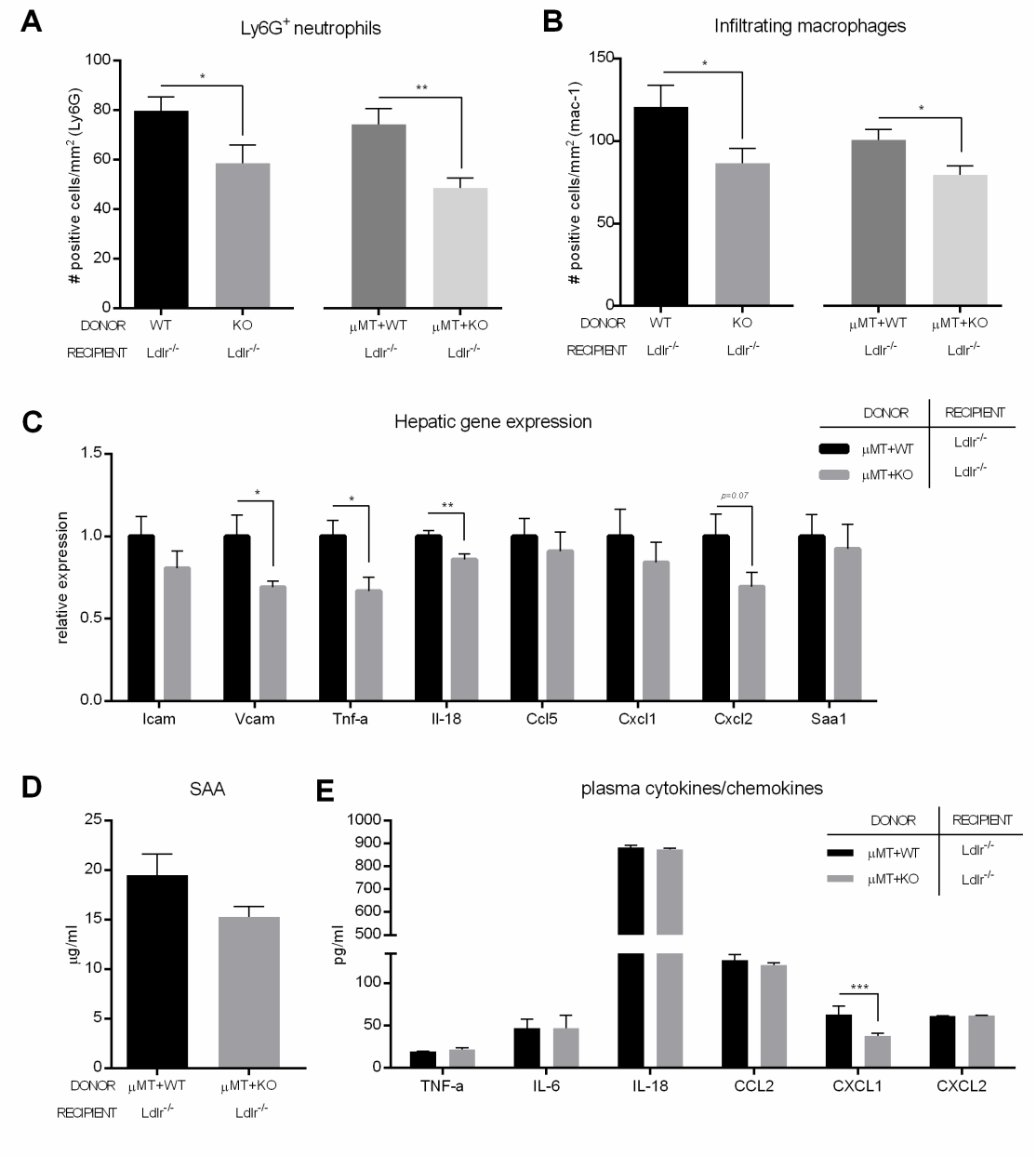

A
Ly6G+ neutrophils
positive cells/mm2 (Ly6G)
DONOR WT KO µMT+WT µMT+KO
RECIPIENT Ldlr-/- Ldlr-/- Ldlr-/- Ldlr-/-
B
Infiltrating macrophages
positive cells/mm2 (mac-1)
C
Hepatic gene expression
relative expression
Icam Vcam Tnf-a Il-18 Ccl5 Cxcl1 Cxcl2 Saa1
p=0.07
D
SAA
µg/ml
E
plasma cytokines/chemokines
pg/ml
TNF-a IL-6 IL-18 CCL2 CXCL1 CXCL2

Figure S6.

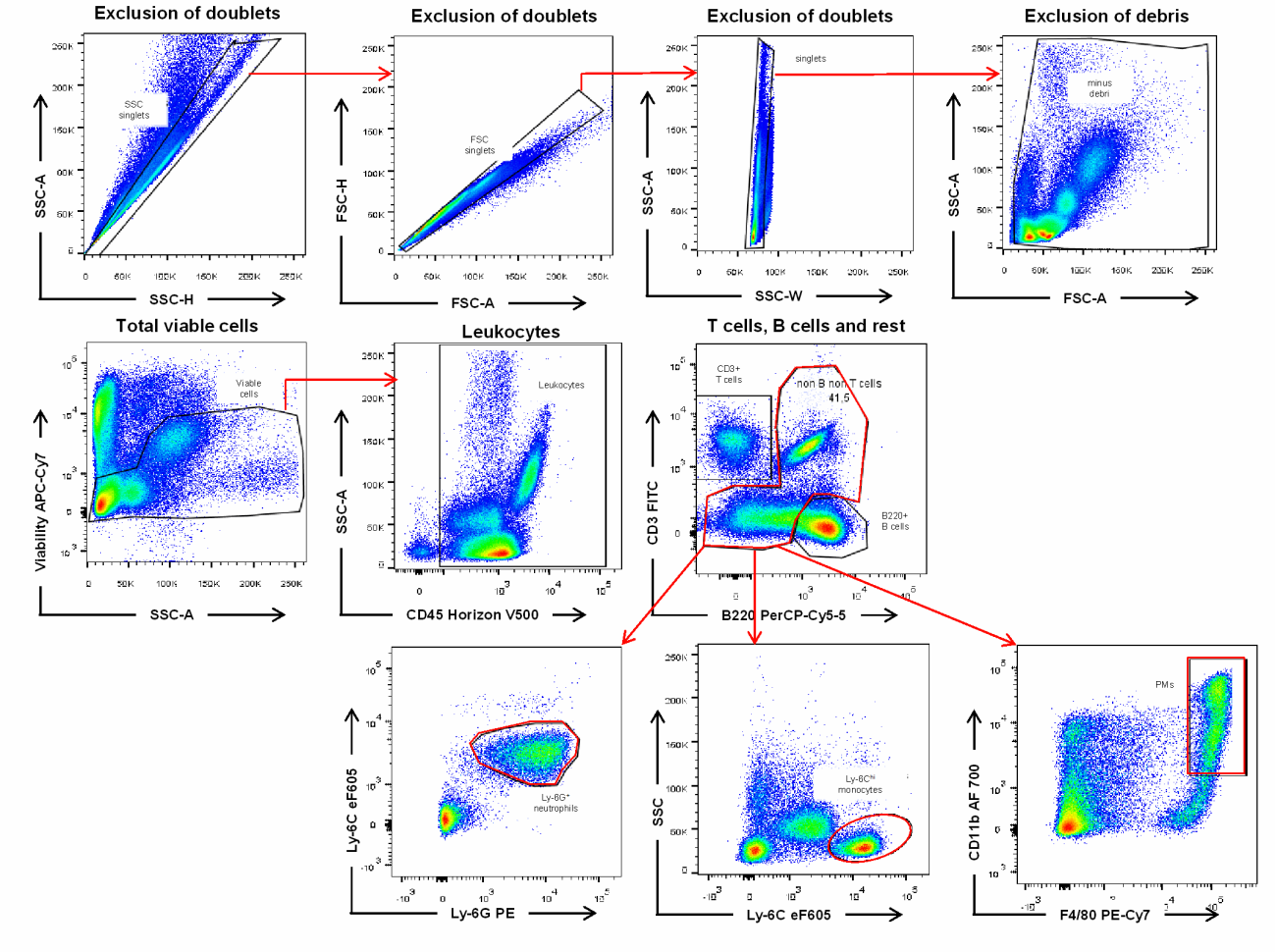
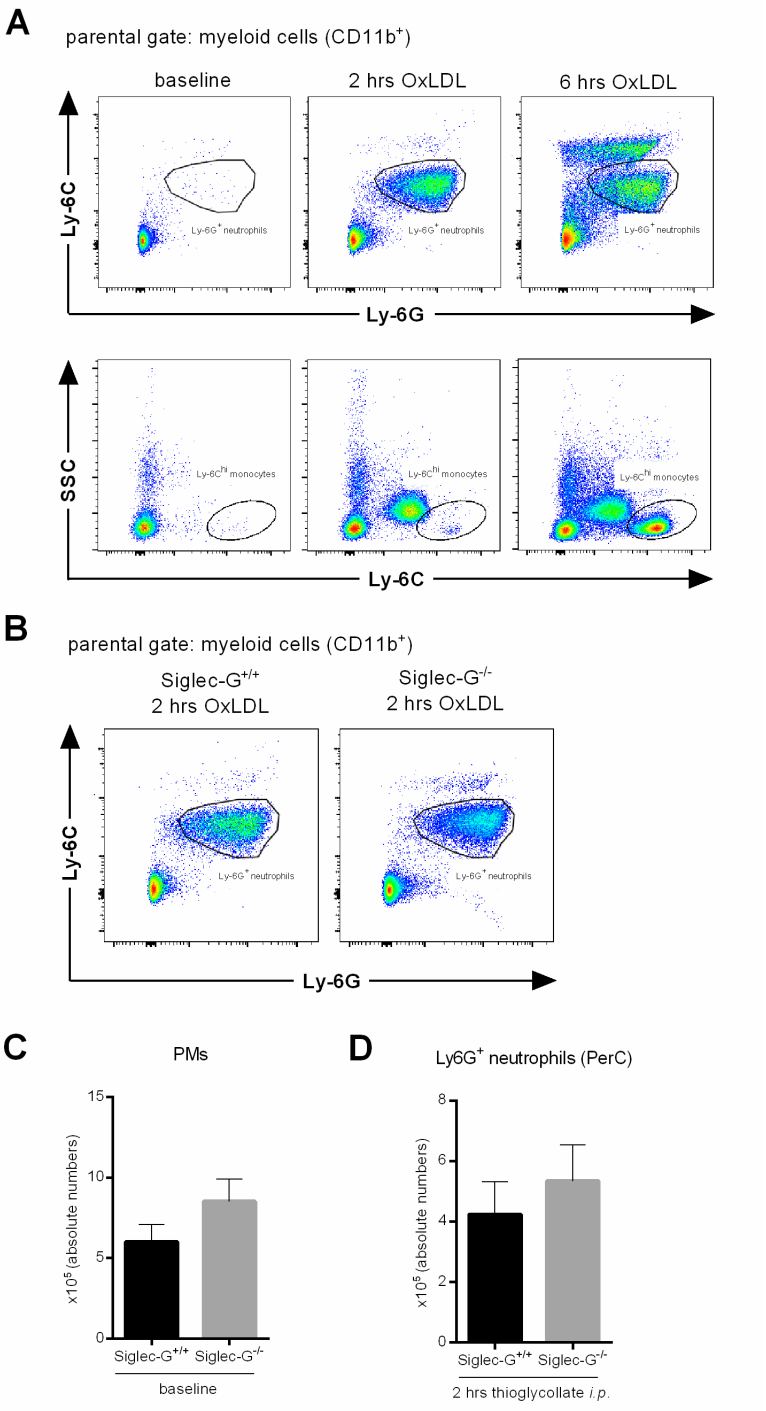

Exclusion of doublets
SSC singlets
SSC-A
SSC-H
Exclusion of doublets
FSC singlets
FSC-H
FSC-A
Exclusion of doublets
singlets
SSC-A
SSC-W
Exclusion of debris
minus debri
SSC-A
FSC-A
Total viable cells
Viable cells
Viability APC-Cy7
SSC-A
Leukocytes
Leukocytes
SSC-A
CD45 Horizon V500
T cells, B cells and rest
CD3+ T cells
non B non T cells 41.5
B220+ B cells
CD3 FITC
B220 PerCP-Cy5-5
Ly-6C eF605
Ly-6G+ neutrophils
Ly-6G PE
SSC
Ly-6C^hi monocytes
Ly-6C eF605
CD11b AF 700
PMs
F4/80 PE-Cy7

Figure S7.


A
parental gate: myeloid cells (CD11b+)
baseline
2 hrs OxLDL
6 hrs OxLDL
Ly-6C
Ly-6G
Ly-6G+ neutrophils
SSC
Ly-6Chi monocytes
B
parental gate: myeloid cells (CD11b+)
Siglec-G+/+
2 hrs OxLDL
Siglec-G-/-
2 hrs OxLDL
C
PMs
x10^5 (absolute numbers)
Siglec-G+/+ Siglec-G-/-
baseline
D
Ly6G+ neutrophils (PerC)
2 hrs thioglycollate i.p.

Supplemental figure legends

Figure S1. Characterization of natural IgM responses in C57BL/6 Siglec-G-deficient mice, Related to Figure 1. (A) Absolute numbers of $B220^{+}IgM^{+}CD43^{+}$ B-1 cells from the spleen. (B) Absolute numbers of $B220^{+}IgM^{+}CD43^{+}CD5^{+}$ B-1a cells from the spleen. (C) Absolute numbers of $B220^{+}CD138^{+}$ plasmablasts from the spleen. (D) Absolute numbers of $B220^{lo}CD138^{+}$ plasma cells from the spleen. (E) Absolute numbers of $B220^{+}CD138^{+}$ plasmablasts from the bone marrow. (F) Absolute numbers of $B220^{lo}CD138^{+}$ plasma cells from the bone marrow. (G) IgM antibody binding to α-1,3-dextran, MDA-LDL, and CuOx-LDL in plasma was determined by ELISA. Plasma was diluted 1:100 and 1:500 and antibody binding was measured in triplicates. Shown are data of *Siglec-G*$^{+/+}$ *and Siglec-G*$^{-/-}$ mice at 8-12 weeks of age. Symbols represent individual mice. Horizontal bars represent the mean and error bars represent SEM of each group. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure S2. Immunological characterization of *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ after 8 weeks of atherogenic diet feeding, Related to Figure 1. (A) Absolute numbers of splenic $CD19^{+}IgM^{hi}CD43^{+}CD5^{+}CD138^{+}$ $CD93^{+}MHCII^{+}$ innate response activator (IRA) B cells. (B and C) Relative numbers of peritoneal cavity $B220^{+}CD5^{-}CD11b^{+}$ B-1b cells (B) and $B220^{+}CD5^{-}CD11b^{-}$ B2 cells (C) out of total B cells. (D) Relative numbers of peripheral blood $B220^{+}$ B cells. (E) Relative numbers of peripheral blood $Ly6C^{+}$ monocytes. (F) Relative numbers of peripheral blood $Ly6C^{+}$ inflammatory and $Ly6C^{lo}$ resident monocytes. (G-H) Titers of MDA-LDL IgG (G) and CuOx-LDL IgG (H) in plasma of *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice were determined by ELISA at baseline and after 8 weeks of atherogenic diet. Plasma samples were diluted 1:100 and 1:500 and antibody binding was measured in triplicates. Symbols represent individual mice (14-16 mice per group). Horizontal bars represent the mean and error bars represent SEM of each group. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure S3. Successful bone marrow reconstitution in *Ldlr*$^{-/-}$ recipient mice, Related to Figure 3. *Ldlr*$^{-/-}$ mice were reconstituted with bone marrow cells isolated from either *Siglec-G*$^{+/+}$ mice or *Siglec-G*$^{-/-}$ [KO] mice or a mixture of 80% bone marrow from *μMT* and 20% either *Siglec-G*$^{+/+}$ [WT] or *Siglec-G*$^{-/-}$ [KO] fed an atherogenic diet for 10 weeks. (A) At time of sacrifice bone marrow cells were collected from mice reconstituted with *Siglec-G*$^{+/+}$ [WT] and *Siglec-G*$^{-/-}$ [KO] and genomic DNA was extracted and amplified for Ldlr and Siglec-G genes. (For some mice DNA could not be recovered successfully). Tail DNA from *Siglec-G*$^{+/+}$, *Siglec-G*$^{-/-}$ and *Ldlr*$^{-/-}$ mice were used as positive and negative controls. (B) Validation of B cell restricted Siglec-G

deficiency in *Ldlr*$^{-/-}$ mice after reconstitution with µMT+WT and µMT+*Siglec-G*$^{-/-}$ [KO] bone marrow after 10 weeks of atherogenic diet. DNA of sorted splenic B (B220^{+}) and non-B cells (B220^{-}) was isolated and pooled samples were analyzed by PCR for the Siglec-G gene. (C) Flow cytometry for Siglec-G expression. Representative histogram of Siglec-G expression on B220^{+} B cells in the spleens of mice reconstituted with µMT+WT and µMT+KO bone marrow after 10 weeks of atherogenic diet.

Figure S4. Immunological characterization of bone marrow chimeric *Ldlr*$^{-/-}$ mice after 10 weeks of atherogenic diet, Related to Figure 3. (A) Relative numbers of peripheral blood Ly6C^{+} monocytes. (B) Relative numbers of peripheral blood Ly6C^{+} inflammatory and Ly6C^{lo} resident monocytes. (C) Relative numbers of peripheral blood B220^{+} B cells. (D) Relative numbers of B220^{+}CD5^{+}CD11b^{+} B-1a cells out of total B cells from the peritoneal cavities. (E) Quantification of total IgG antibodies in plasma. Plasma samples were diluted 1:70,000 and measured in triplicates. (F-G) Binding of IgG antibodies to (F) MDA-LDL and (G) CuOx-LDL was determined by ELISA. Plasma samples were diluted between 1:100 and 1:500 and antibody binding was measured in triplicates. Data are expressed as RLU per 100 ms. Symbols represent individual mice. (H) IgM antibody repertoire to model epitopes of OxLDL. Titers of α-1,3-dextran IgM, MDA-LDL IgM and CuOx-LDL IgM in plasma were determined by ELISA. Plasma was diluted 1:100 and 1:500 and antibody binding was measured in triplicates. The data is plotted as the ratio of antigen-specific IgM to total IgM. Shown are data of *Ldlr*$^{-/-}$ mice reconstituted with *Siglec-G*$^{+/+}$ (n = 10) vs. *Siglec-G*$^{-/-}$ [KO] (n = 11) and µMT+*Siglec-G*$^{+/+}$ (n = 13) vs. µMT+*Siglec-G*$^{-/-}$ [KO] (n = 13) bone marrow after 10 weeks of atherogenic diet. Horizontal bars represent the mean and error bars represent SEM of each group. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure S5. Selective Siglec-G deficiency in B cells protects from atherogenic diet-induced hepatic and systemic inflammation, Related to Figure 4. (A, B) Quantification of infiltrating neutrophils (A) and macrophages (B) in liver sections of *Ldlr*$^{-/-}$ mice reconstituted with *Siglec-G*$^{+/+}$ [WT] vs. *Siglec-G*$^{-/-}$ [KO] and µMT+*Siglec-G*$^{+/+}$ [WT] vs. µMT+*Siglec-G*$^{-/-}$ [KO] and fed with an atherogenic diet for 10 weeks. Sections were stained with anti-Ly6G and anti-mac-1 antibody, and positively stained cells were counted and expressed as number of positive cells per mm^2. (C) Relative gene expression of *Icam, Vcam, Tnf-α, Il-18, Ccl5, Cxcl1, Cxcl2* and *Saa1* mRNA in livers of *Ldlr*$^{-/-}$ mice reconstituted with µMT+*Siglec-G*$^{+/+}$ [WT] vs. µMT+*Siglec-G*$^{-/-}$ [KO] bone marrow and fed an atherogenic diet for 10 weeks. The expression of individual genes was

normalized to the house-keeping gene S12 and expressed relatively to the expression in µMT+*Siglec-G*$^{+/+}$ [WT] control mice. (D) Quantification of SAA levels in plasma of *Ldlr*$^{-/-}$ mice reconstituted with µMT+*Siglec-G*$^{+/+}$ [WT] vs. µMT+*Siglec-G*$^{-/-}$ [KO] bone marrow and fed an atherogenic diet for 10 weeks. Plasma samples were diluted 1:300 and measured in triplicates. (E) Quantification of a panel of chemokines and cytokines in plasma of *Ldlr*$^{-/-}$ mice reconstituted with µMT+*Siglec-G*$^{+/+}$ [WT] vs. µMT+*Siglec-G*$^{-/-}$ [KO] bone marrow and fed an atherogenic diet for 10 weeks by multiplex assay. Plasma samples were diluted 1:2. Data represent mean ± SEM of 9-13 mice per group. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure S6. Representative flow cytometry plots and gating strategy for analysis of peritoneal cell populations upon sterile peritonitis induction, Related to Figure 5. *Siglec-G*$^{+/+}$ and *Siglec-G*$^{-/-}$ mice were injected *i.p.* with OxLDL (25 µg/g body weight) and peritoneal exudate cells were harvested to monitor infiltration of immune cells. Representative flow cytometry plots and gating strategy is shown: After exclusion of doublets and cell debris by FSC and SSC, viable cells were identified by staining with a viability dye. CD45 was used to separate individual cell populations. T cells and B cells were identified by CD3 and B220 markers, respectively. The “non T and non B cells” gate (CD3$^-$B220$^-$ cells) was further used to define Ly-6G$^+$ neutrophils, Ly-6C^{hi} monocytes and CD11b$^+$F4/80$^+$ macrophages.

Figure S7. Characterization of sterile peritonitis in Siglec-G-deficient mice, Related to Figure 5. (A) Representative flow cytometry plots for Ly6G$^+$ neutrophils and Ly6G^{hi} inflammatory monocytes of C57BL/6 mice are shown at baseline, as well as 2, 6, and 12 hours after OxLDL injection. (B) Representative flow cytometry plots for Ly6G$^+$ neutrophils in the PLF of *Siglec-G*$^{+/+}$ vs. *Siglec-G*$^{-/-}$ mice 2 hours after *i.p.* OxLDL injection are shown. (C) Absolute numbers of CD11b$^+$F4/80$^+$ peritoneal macrophages (PMs) in the peritoneal lavage fluid at baseline in *Siglec-G*$^{+/+}$ and *Siglec-G*$^{-/-}$ mice. (D) Absolute numbers of Ly6G$^+$ neutrophils in the peritoneal lavage fluid after 2 hours of thioglycollate injection (25 µl/g body weight, *i.p.*) in *Siglec-G*$^{+/+}$ and *Siglec-G*$^{-/-}$ mice. Data represent mean and error bars represent SEM of 3-7 mice per group. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Supplemental experimental procedures

Bone marrow transplantation

Bone marrow transplantation studies were performed as previously described (Binder et al., 2003; Fillatreau et al., 2002; Sage et al., 2012). Ten weeks-old male *Ldlr*$^{-/-}$ mice received a single dose of 9.5 Gy lethal irradiation and were subsequently injected intravenously via the retro-orbital plexus with 1×10^7 bone marrow cells from either *Siglec-G*$^{-/-}$ ($n = 12$) or *Siglec-G*$^{+/+}$ ($n = 12$) mice. In a separate experiment in order to assess the effects of selective deficiency of Siglec-G on B cells, irradiated *Ldlr*$^{-/-}$ mice were reconstituted with a mixture of 80% bone marrow from *μMT* mice (no B cells due to disruption of the membrane exon of the mu heavy chain gene) and 20% bone marrow from either *Siglec-G*$^{+/+}$ ($n = 13$) or *Siglec-G*$^{-/-}$ ($n = 13$) mice. After 4 weeks of recovery, mice were fed an atherogenic diet containing 21% milk fat and 0.2% cholesterol (Ssniff Spezialdiäten GmbH, Soest, Germany) for 10 weeks to induce lesion formation. Three mice (two in the *Siglec-G*$^{+/+}$ and one in *Siglec-G*$^{-/-}$) died during the study.

Evaluation of atherosclerosis

The extent of atherosclerosis was assessed in the entire aorta and in cross-sections of the aortic origin, as previously described (Cardilo et al., 2012). Histological sections of the paraffin-embedded aortic origin were stained with a modified elastic-trichrome stain for quantification of lesion size and size of necrotic areas. Macrophage areas were assessed by immunohistochemistry using anti-mouse-mac-3 antibodies (rat anti-mouse, clone M3/84, BD-Biosciences Pharmingen, San Diego, California, USA). For the quantification of lesional collagen content, sections were stained with Sirius Red (Direct Red 80, Sigma Aldrich). For the presence of IgM, immunohistochemistry was performed using an antibody against mouse IgM, μ-chain specific (Sigma-Aldrich). For indicated experimental studies, innominate arteries were isolated and embedded in paraffin as described (Reardon et al., 2003). All photographed images were quantified using Adobe Photoshop CS5 and analyzed using ImageJ 1.47 software.

Flow cytometry

At time of sacrifice, peritoneal exudates cells (PECs) were collected by lavaging the peritoneum with 10 ml of sterile HBSS + 2% FBS. PECs were pelleted; cells were counted and subsequently processed for flow cytometry. Spleens and bone marrow were harvested and passed through a cell strainer to obtain a single cell suspension and red blood cells were lysed using a commercial lysis buffer (Morphisto). Total white blood cells were isolated from blood

collected in EDTA-tubes via the vena cava. Blood was diluted 1:1 in a solution of PBS containing 2% Dextran (Sigma-Aldrich) and incubated for 40 min at 37ºC to separate the red blood cells. The supernatant was harvested and used for flow cytometry analysis. 1 × 10^6 cells were used for flow cytometry staining, and 5 × 10^6 cells were lysed in 350 µl RLT RNA lysis buffer (Qiagen) and stored at -20°C.

For flow cytometric analyses, cells were blocked with anti-mouse CD16/32 blocking Ab (anti-mouse CD16/CD32, clone 93, 0.5 µg/0.5 × 10^6 cells, eBioscience) for 20 min at 4°C and stained for 30 min with following antibodies: PerCP-Cy5.5-labeled anti-CD45R (B220) (clone RA3-6B2, 1:800, eBioscience), FITC-labeled anti-CD23 (clone B3B4, 1:600, BD Biosciences – Pharmingen), APC-labeled anti-IgM (clone II/41, 1:600, eBioscience), phycoerythrin (PE)-labeled anti-CD43 (clone S7, 1:600, BD Biosciences – Pharmingen), biotin-labeled anti-CD21/CD35 (CR2/CR1) (clone 7E9, 1:200, BioLegend), PE-labeled anti-CD3e (clone 145-2C11, 1:400, eBioscience), FITC-labeled anti-CD4 (clone GK1.5, 1:400, eBioscience), APC-labeled anti-CD8a (clone 53-6.7, 1:400, eBioscience), FITC-labeled anti-kappa (clone 197.1, 1:400, BD Biosciences – Pharmingen), Biotin-labeled anti-lambda (clone RML-42, 1:400, BioLegend), PE-labeled anti-IgD (clone 11-26c (11-26), 1:200, eBioscience), PE-labeled anti-CD93 (clone AA4.1, 1:400, eBioscience), APC-labeled anti-Siglec-G (clone SH2.1, 1:400, eBioscience), APC-labeled anti-CD11b/Mac-1 (clone M1/70, 1:600, eBioscience), FITC-labeled anti CD11b (clone M1/70, 1:600, eBioscience), PE-labeled anti-CD5 (clone 53-7.3, 1:100, eBioscience), FITC-labeled anti-Ly6C (clone HK14, 1:200, BioLegend), PE-labeled anti-Ly6G (clone 1A8, 1:2000, BioLegend), V500-labeled anti-CD45 (clone 30-F11, 1:200, BD Biosciences – Pharmingen), BV570-labeled anti-Ly-6C (clone HK1.4, 1:100, BioLegend), eFluor 780-labeled fixable viability dye (1:1000, eBioscience), AF700-labeled anti-CD11b (clone M1/70, 1:700, eBioscience), Brilliant Violet 570-labelled anti-CD19 (1:100, clone 6D5, BioLegend), PE-labelled anti-GM-CSF (1:100, clone MP1-22E9, BioLegend), PECy7-labelled anti CD93 (1:100, clone AA4.1, eBioscience), biotin-labeled anti-CD5 (Ly-1) (1:200, clone 53-7.3, BD Pharmingen), AF700-labelled anti-MHCII (1:100, clone M5/114.15.2, eBioscience), FITC-labeled anti-IgD (1:800, clone 11-26c, eBioscience), biotin-labeled anti-CD138 (Syndecan 1) (1:200, clone 281-2, BioLegend). All stains were performed in 100 µl of FACS buffer (PBS + 10% FCS) for 30 min at 4°C in darkness, followed by two washing steps. Stained cell populations were analyzed by multiparameter flow cytometry using a BD FACSCalibur (BD Bioscience, Franklin Lakes, New Jersey, USA) or BD FACS Fortessa, respectively. Either 1×10^5 or 1×10^6 cells per sample were stained and acquired. Dead cells and doublets were excluded by forward- and side-scatter and

data were analyzed using the FlowJo version 10 data analysis software (Tree Star Inc., Oregon Corporation, Ashland, USA).

Clinical chemistry and lipid analyses

At time of sacrifice, blood was collected via the vena cava into EDTA tubes (MiniCollect® 1 ml K_3EDTA Blood Collection Tube, Greiner Bio-One), centrifuged for 30 min at 1000 rpm, and aliquots of plasma were stored at -80°C for further analyses. Total plasma cholesterol and triglycerides were measured by enzymatic methods using an automated analyzer AU5400 – Chemistry System (Beckman Coulter, Brea, California, USA). Liver lipid levels were measured by enzymatic methods using the CHOD-PAP assay (Roche Diagnostics, Basel, Switzerland) for cholesterol and a triglyceride determination kit (Sigma-Aldrich). Non-esterified fatty acids (NEFA) in the liver were determined using the NEFA-C kit (ACS-ACOD method; Wako Chemicals, Neuss, Germany). All measurements were done according to the manufacturer's protocols using a Benchmark 550 Micro-plate Reader (Bio-Rad, Hercules, CA). Protein content was measured with the bicinchoninic acid (BCA) method (Pierce, Rockford, IL) according to manufacturer's protocol. Data are expressed as µg lipid per µg protein.

ALT levels were measured in plasma (30 µl, 1:3 diluted in physiological NaCl) of each individual mouse by use of the Reflotron system in combination with the strips for alanine aminotransferase (Roche Diagnostics, Almere, The Netherlands) according to the manufacturer's instructions.

Gene expression analysis

Liver. Total RNA was isolated from mouse liver tissue and was reversely transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, USA). Quantitative real-time PCR was performed using SensiMix™ SYBR green with ROX dye (Bioline*,* Randolph, USA) on an ABI7900HT instrument (Applied Biosystems). The relative expression level of target genes was determined with the LinRegPCR software (http://www.hfrc.nl, (Ruijter et al., 2009), and obtained values were normalized to the housekeeping gene ribosomal protein S12 (Rps12). Values are expressed as the relative expression compared to the control group.

Primer sequences:

Cxcl2-forward: 5'-AGTGAACTGCGCTGTCAATGC-3';
Cxcl2-reverse: 5'-AGGCAAACTTTTTGACCGCC-3';
TNF-α-forward: 5'-CATCTTCTCAAAATTCGAGTGACAA-3';
TNF-α-reverse: 5'-TGGGAGTAGACAAGGTACAACCC-3';

Cxcl1-forward: 5'-TGCACCCAAACCGAAGTCAT-3';
Cxcl1-reverse: 5'-TTGTCAGAAGCCAGCGTTCAC-3';
SAA1-forward: 5'-GGCTGCTGAGAAAATCAGTGATG-3';
SAA1-reverse: 5'-TCAGCAATGGTGTCCTCATGTC-3';
VCAM-forward: 5'-GTGTTGAGCTCTGTGGGTTTTG-3';
VCAM-reverse: 5'-TTAATTACTGGATCTTCAGGGAATGAG-3';
ICAM-forward: 5'-CTACCATCACCGTGTATTCGTTTC-3';
ICAM-reverse: 5'-CGGTGCTCCACCATCCA-3';
IL-18-forward: 5'-ACAACTTTGGCCGACTTCAC-3';
IL-18-reverse: 5'-GGGTTCACTGGCACTTTGAT-3';
CCL5-forward: 5'-GGAGTATTTCTACACCAGCAGCAA-3';
CCL5-reverse: 5'-GCGGTTCCTTCGAGTGACA-3';
S12-forward: 5'-GGAAGGCATAGCTGCTGGAGGTGT-3';
S12-reverse: 5'-CCTTCGATGACATCCTTGGCCTGAG-3'.

Validation of successful BMT

At time of sacrifice, bone marrow cells from all mice were collected and genomic DNA was extracted by incubating cells in 40 µl of lysis buffer (1 M Tris/HCl, pH 8.0, 500 mM EDTA, 10% SDS, 2 M NaCl, 0.4 mg/ml Proteinase K) at 55°C overnight with gentle mixing, followed by 10 min incubation at 99°C to inactivate Proteinase K. The purified genomic DNA was diluted 1:10 in H_2O and amplified by PCR for the Ldlr and Siglec-G gene to identify successful bone marrow reconstitution with donor bone marrow. In order to assess selective Siglec-G deficiency on B cells, B cells and non B cells of splenocytes from recipient mice were separated using anti-CD45R (B220) MicroBeads (Milteny Biotec) according to the manufacturer's protocol. Collected cell fractions were lysed in RLT Plus buffer (Qiagen) and stored at -20°C. Genomic DNA was purified with the AllPrep DNA/RNA Micro Kit (Qiagen) according to the manufacturer's protocol and equal amounts (50 ng) were used for PCR to amplify the genes of interest.

Primer sequences:

Siglec-G-forward: 5'-CCGCTCGAGATGTTGTCCCGCGGGTGGTTTCA-3';
Siglec-G-reverse: 5'-CCGGAATTCCTTCCTCTTGAGAGAACCTTTGTTC-3';
Ldlr-forward: 5'-ACCCCAAGACGTGCTCCCAGGATG-3';
Ldlr-reverse: 5'-CGCAGTGCTCCTCATCTGACTTGT-3';
neo cassette primer: 5'-AGGTGAGATGACAGGAGA-3'.

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3. DISCUSSION

In this thesis, I report a pathogenic role for Siglec-G in the context of atherosclerosis and liver inflammation. I demonstrate that Siglec-G deficiency in high fat diet-fed *Ldlr*$^{-/-}$ mice results in a profound reduction of atherosclerotic lesion size as well as to diminished liver inflammation. Furthermore, I have shown that these protective effects are mediated by Siglec-G deficiency selectively in B cells. Consequently, Siglec-G deficiency leads to elevated B-1 cells and a robust and preferential increase in OSE-specific natural IgM antibodies, which have the capacity to neutralize OxLDL-induced inflammation *in vivo*.

Siglec-G has been shown to be primarily expressed on B cells, and in this regard higher expression levels could be observed on B-1a cells compared to conventional B2 cells (Hoffmann et al, 2007). However, Siglec-G has also been shown to be expressed on other cells than B cells, being dendritic cells and eosinophils (Hutzler et al, 2014; Pfrengle et al, 2013). It has been suggested that Siglec-G in non B cells exerts a suppressive role in the context of drug-induced liver damage. In more detail, Siglec-G deficiency leads to an increased production of the pro-inflammatory cytokines IL-6, TNF-α, and MCP-1, that are released when stimulated with DAMPs (Chen et al, 2009). Regardless, my data prominently describe anti-inflammatory effects of Siglec-G deficiency in high fat diet-fed *Ldlr*$^{-/-}$ mice as observed by diminished expression of adhesion molecules, pro-inflammatory cytokines and chemokines in the liver as well as reduced levels of CXCL1 in the plasma. In addition, the data reported herein in this thesis make evident that Siglec-G deficiency exerts protective effects in a model of OxLDL-induced sterile peritonitis, although deficiency of Siglec-G exhibited aggravated inflammation in cecal ligation and puncture (CLP)-induced polybacterial peritonitis model. These adverse effects mentioned above have been demonstrated to be dependent on the interaction of Siglec-G with the GPI-anchored co-stimulatory protein CD24 (Chen et al, 2011). In this context, CD24 recognizes DAMPs and thereby allows Siglec-G to suppress pro-inflammatory signaling via PRRs, which can be disrupted by bacterial sialidases but not by LPS.

In my thesis I have demonstrated that deficiency of Siglec-G exerts protection against OxLDL, which represents a different type of sterile trigger. In more detail, I could show in experimental models of OxLDL-induced sterile peritonitis that mice deficient in Siglec-G show reduced inflammation, as seen by decreased inflammatory cell recruitment as well as reduced levels of pro-inflammatory cytokines in the peritoneal lavage fluids compared to the control mice. One

can assume that sterile inflammation induced by OxLDL does not interfere with the interaction of Siglec-G and CD24, proposing that Siglec-G deficiency confers its protective effects via other mechanisms than CD24. Moreover, it has been demonstrated that RNA viruses have the ability to evade anti-viral defenses by up-regulating Siglec-G in macrophages and dendritic cells. This consequently leads to a diminished production of type I interferons that are crucial for the host defense against viruses (Chen et al, 2013). As a result, deficiency of Siglec-G leads to an elevated production of type I interferons, which of note have been shown to play pro-atherogenic roles (Ait-Oufella et al, 2011). Due to the fact that I have demonstrated in this thesis that Siglec-G deficiency acts protective with respect to atherosclerosis, this pathway seems to be irrelevant in this condition. Consistent with this, I have observed in *in vitro* experiments that the stimulation of peritoneal as well as bone-marrow derived macrophages with OxLDL does not induce the expression of Siglec-G (data not shown). Collectively, all this data indicates against a role of Siglec-G in myeloid cells in the context of atherosclerosis and liver inflammation.

Furthermore, I provide convincing evidence that the protective effects of Siglec-G deficiency are mediated specifically by Siglec-G deficiency on B cells, which argues that the function of Siglec-G on B cells is dominant in this setting. As mentioned above, the expression of Siglec-G occurs throughout all stages in B cell development (Hutzler et al, 2014; Pfrengle et al, 2013). Hence, the functional role of Siglec-G in the context of autoimmunity has been of interest. Mueller *et al.* have demonstrated that ageing *Siglec-G*$^{-/-}$ mice show signs for an over-activation status of the adaptive immune system as increased expression of activation markers on T cells as well as on conventional B2 cells was observed (Muller et al, 2015). Nonetheless, the protective effects presented in this thesis are more consistent with the expanded production of protective B-1 cell-derived natural antibodies by Siglec-G-deficient mice.

Consistent with the previous data obtained, I also show that Siglec-G deficiency results in a marked expansion of the peritoneal B-1a population as well as splenic B-1 cells in hypercholesterolemic mice. Both B2 cell populations, FO B cells and MZB cells, are not different in *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice as well as in the bone marrow chimeric mice compared to the controls. Therefore, these data further support an atheroprotective role for B-1 cells (Tsiantoulas et al, 2014). Interestingly, I have shown a profound increase of splenic IRA B cells in atherosclerotic Siglec-G-deficient *Ldlr*$^{-/-}$ mice. IRA B cells have been suggested to stem from peritoneal B-1a cells and have been shown to play a pro-atherogenic role (Hilgendorf *et al.*, 2014). Along this

line, my data are consistent with a B-1 cell origin of IRA B cells and importantly B-1 cells and their protective capacity predominate in atherosclerotic mice which lack Siglec-G.

Experimental transfer studies have revealed an anti-atherogenic effect of adoptively transferred B-1a cells into splenectomized *Apoe*$^{-/-}$ mice and of B-1b cells into *Rag-1*$^{-/-}$*Apoe*$^{-/-}$ mice (Kyaw et al, 2011; Rosenfeld et al, 2015). Notably, these investigations are directed towards the role of B-1 cells in immune-compromised mice with additional existing differences in various immune cell populations, such as the lack of splenoctyes or total lymphocytes as a result of splenectomy or Rag-1 deficiency, respectively. Thus, these studies do not provide a conclusive answer on the role of B-1 cells in atherosclerosis in an intact host. Importantly, the data of this thesis demonstrates for the first time clear and direct genetic evidence that the expansion of B-1a cells in intact mice confers protection in the context of atherosclerosis.

The major function of B-1 cells is to produce natural IgM antibodies, which in turn have been proposed to exert the atheroprotective effects of B-1 cells. For example, Kyaw *et al*. demonstrated that accelerated atherosclerosis in splenectomized *Ldlr*$^{-/-}$ mice, which have reduced peritoneal B-1a cell numbers and lower IgM antibodies in the serum, is reversed by the adoptive transfer of B-1a cells. It was shown that this effect is dependent on the capacity of B-1 a cells to secrete natural IgM antibodies, as adoptive transfer of B-1 cells from secreted *IgM*$^{-/-}$ donors (mice which cannot secrete natural IgM antibodies) did not show any effect (Kyaw et al, 2011). A protective role of natural IgM antibodies is further affirmed by studies conducted in *Ldlr*$^{-/-}$ mice which are on top unable to secrete IgM. These mice develop aggravated atherosclerosis compared to the respective control mice (Lewis et al, 2009). Moreover, these data lead to the hypothesis that increasing IgM titers beyond physiological levels could mediate protection against atherosclerosis.

Notably, it has been demonstrated that hypercholesterolemia increases plasma IgM titers and it is speculated that this occurs as an endogenous defense mechanisms towards increased accumulation of metabolic waste products (Khoo et al, 2015). Nonetheless, this response is not capable of preventing atherosclerosis and liver inflammation. Of note, Siglec-G deficiency leads to a more profound elevation of natural IgM antibodies despite excessive hypercholesterolemia. Taken this into consideration, I believe that Siglec-G could serve as an attractive target, e.g. by developing blocking antibodies which could be used for therapeutic intervention.

Natural IgM antibodies possess protective properties by exerting house-keeping functions and promoting the clearance of dying cells and cellular debris (Avrameas, 1991; Notkins, 2004). These functions of natural IgM antibodies are partly conferred by their ability to recognize OSE, which are also present on OxLDL (Miller et al, 2011). My thesis laboratory has demonstrated that immunization of high fat diet-fed *Ldlr*$^{-/-}$ mice with heat-killed pneumococci leads to the expansion of a natural IgM clone called T15/E06, which recognizes OxLDL. Importantly, this consequently results in significantly diminished atherosclerosis as well as liver inflammation (Bieghs et al, 2012b; Binder et al, 2003). Taken together, these data indicate that IgM antibodies targeted against OxLDL protect from atherosclerosis and hepatic inflammation. Consistently, epidemiological studies in humans have associated high levels of OxLDL-specific IgM with a lower risk for developing CVD (Tsiantoulas et al, 2014). Notably, I could demonstrate that Siglec-G deficiency results in a preferential increase of IgM antibodies with specificity for epitopes of OxLDL in plasma and protects from atherosclerosis. In this regard, several mechanisms exist by which IgM antibodies specific for OxLDL exhibit protection against atherosclerosis. One of these mechanisms includes the ability of natural IgM to inhibit foam cell formation by blocking scavenger receptor-mediated uptake of OxLDL by macrophages (Tsiantoulas et al, 2014). Furthermore, IgM antibodies specific for OxLDL are capable of preventing the accumulation of apoptotic cells by promoting their uptake by macrophages (Chou et al, 2009; Ogden et al, 2005). Consistent with this, I demonstrate smaller necrotic areas in the aortic root in lesions of high fat diet-fed *Ldlr*$^{-/-}$*Siglec-G*$^{-/-}$ mice. In addition, OxLDL-specific IgM antibodies have been shown to inhibit inflammation by components of OxLDL *in vitro* (Imai et al, 2008). In line with a neutralizing capacity of OxLDL-specific IgM, I report diminished secretion of the cytokines CXCL1 and CXCL2 as well as reduced neutrophil infiltration into the peritoneal cavities of Siglec-G-deficient mice 2 hours after *i.p.* injection of OxLDL. Notably, *Siglec-G*$^{-/-}$ mice have more than 9-fold elevated levels of OxLDL-specific IgM antibodies in the peritoneal cavity compared to controls. Additionally, levels of CXCL1 in the plasma are significantly reduced in high fat diet-fed Siglec-G-deficient mice. Interestingly it is known that OxLDL induces the expression of Cxcl1 in macrophages via a co-operation of CD36/TLR-4/TLR-6, and represents a key mediator of leukocyte recruitment in atherosclerosis (Stewart et al, 2010). Furthermore, the expression of CD36 and TLR-4 in macrophages has been shown to promote atherosclerosis and liver inflammation.

Hence, elevated natural IgM antibodies directed against OxLDL in Siglec-G-deficient mice possess the capacity to inhibit atherosclerosis and hepatic inflammation by neutralizing the pro-inflammatory properties of OxLDL.

3.1. Conclusion & future prospects

In this thesis, I provide for the first time evidence that expansion of natural IgM antibodies beyond and above physiological plasma levels protect from atherosclerosis and liver inflammation.

I believe that exploiting these mechanisms, e.g. by blocking the inhibitory effect of Siglec-G, represent a novel therapeutic approach to enhance and strengthen the endogenous defense mechanisms in order to protect from cardiovascular disease and associated metabolic disorders, such as non-alcoholic steatohepatitis.

4. MATERIALS & METHODS

4.1. Mice

LDL receptor-deficient mice (*Ldlr*$^{-/-}$) were purchased originally from The Jackson Laboratories (Bar Harbor, Maine, USA); the generation of *Siglec-G*$^{-/-}$ mice has been described elsewhere (Mueller et al., 2015). *Siglec-G*$^{-/-}$ mice, which were originally on a BALB/c background, were first backcrossed to a C57BL/6 background for 5-6 generations using a genome microsatellite marker-assisted speed congenic method *(*ABI PRISM® Mouse Mapping Primers v.1.0, Applied Biosystems, Carlsbad, USA*)* and crossed with *Ldlr*$^{-/-}$ mice to obtain *Siglec-G*$^{-/-}$*Ldlr*$^{-/-}$ and *Siglec-G*$^{+/+}$*Ldlr*$^{-/-}$ mice. *μMT* mice were on a C57BL/6 background and a kind gift of Dr. Ziad Mallat (Department of Medicine, University of Cambridge). All experimental protocols were approved by the institutional animal experimentation committee and the Austrian Ministry of Science.

4.2. Diet intervention studies

For atherosclerosis studies, 12 weeks-old male *Siglec-G*$^{-/-}$*Ldlr*$^{-/-}$ mice (n = 16) and *Siglec-G*$^{+/+}$*Ldlr*$^{-/-}$ littermate controls (n = 14) were fed an atherogenic diet containing 21% milk fat and 0.2% cholesterol (TD88137, Ssniff Spezialdiäten GmbH, Soest, Germany) for 8 weeks to induce lesion formation. To assess the role of Siglec-G deficiency on hematopoetic cells and B cells specifically, bone marrow transplantation (BMT) studies were performed as previously described (Binder et al, 2003; Fillatreau et al, 2002; Sage et al, 2012). Ten weeks-old male *Ldlr*$^{-/-}$ mice received a single dose of 9.5 Gy lethal irradiation and subsequently injected intravenously via the retro-orbital plexus with 1×10^7 bone marrow cells from either *Siglec-G*$^{-/-}$ (n = 12) or *Siglec-G*$^{+/+}$ (n = 12) mice. In order to assess the effects of selective deficiency of Siglec-G on B cells, irradiated *Ldlr*$^{-/-}$ mice were reconstituted with a mixture of 80% bone marrow from *μMT* mice (which do not develop B cells due to disruption of the membrane exon of the mu heavy chain gene) and 20% bone marrow from either *Siglec-G*$^{+/+}$ (n = 13) or *Siglec-G*$^{-/-}$ (n = 13) mice. After 4 weeks of recovery, mice were fed an atherogenic diet containing 21% milk fat and 0.2% cholesterol (Ssniff Spezialdiäten GmbH, Soest, Germany) for 10 weeks to induce lesion formation.

Blood of all experimental mice was obtained before the onset of high-fat diet feeding as well as at an intermediate time-point (4 weeks HFD) by bleeding via the tail vein into heparinized glass capillary tube (Brand GmBH + CoKG, Wertheim, Germany), and plasma aliquots were

frozen at -80°C for further analyses. At time of sacrifice, peritoneal exudates cells (PEC) were collected by lavaging the peritoneum with 10 ml of sterile HBSS + 2% FBS. PEC were pelleted for 10 min at 300 g, and cells were counted and subsequently processed for flow cytometry. Blood was collected via the vena cava into EDTA tubes (MiniCollect® 1 ml K_3EDTA Blood Collection Tube, Greiner Bio-One) and aliquots of plasma were stored at -80°C for further analyses. To isolate peripheral blood leukocytes, an aliquot of blood was mixed at a 1:1 ratio with PBS containing 2% Dextran (Sigma-Aldrich) and incubated for 40 min at 37ºC to separate red blood cells. Subsequently, leukoctyes were washed and processed flow cytometry. Spleens were harvested and passed through a cell strainer to obtain a single cell suspension and red blood cells were lysed using a commercial lysis buffer (Morphisto). An aliquot of 1×10^6 cells were used for flow cytometry staining, and 5×10^6 cells were lysed in 350 µl RLT RNA lysis buffer (Qiagen) and stored at -20°C. The left lobe of the liver was removed and divided into 4 equal pieces, which were transferred into screw-cap microtubes (RNA isolation, liver lipid measurements, histology and reserve) and snap frozen in liquid nitrogen. Bones from the hind legs (femur and tibia) were used to isolated bone marrow cells. The tips of the bones (tibia at distal end, greater trochanter at femur) were removed and placed in microtubes with perforated bottoms. Following centrifugation at 13,000 rpm for 2 min at 4°C, red blood cells were lysed and cells were resuspended in100 µl lysis buffer (1 M Tris/HCl, pH 8.0, 500 mM EDTA, 10% SDS, 2 M NaCl, 0.4 mg/ml Proteinase K).

4.3. Evaluation and phenotypic analysis of atherosclerotic lesions

The extent of atherosclerosis was assessed in a blinded fashion in *en face* preparations of the entire aorta and in cross-sections through the aortic origin by computer-assisted image analysis as previously described (Binder et al, 2004; Schiller et al, 2001). Histological sections of the paraffin-embedded aortic origin were stained with a modified elastic-trichrome stain for quantification of lesion size and size of necrotic areas. Macrophage areas were assessed by immunohistochemistry using anti-mouse-mac-3 antibodies (1:50 rat anti-mouse, clone M3/84, BD-Biosciences Pharmingen, San Diego, California, USA). For indicated experimental studies, the innominate arteries were isolated and embedded in paraffin as described (Reardon et al, 2003). Sequential sections of 5 µm thickness were obtained between 150 µm to 450 µm from the branch point of the innominate artery to the aortic arch, and lesion size in the innominate artery was quantified in 4 sections separated by 100 µm after staining with a modified elastic

trichrome stain. All photographed images were quantified using Adobe Photoshop CS5 and analyzed using ImageJ 1.47 software.

4.4. Liver histology

Immunohistochemical stainings for inflammatory markers were performed on frozen liver sections (7 µm). In summary, frozen liver sections were fixed in acetone and subsequently blocked for endogenous peroxidase by incubation with 0.25% of 0.03% H_2O_2 for 5 min. After blocking with 4% fetal calf serum in PBS for 30 min, sections were stained for the presence of infiltrating macrophages and neutrophils by incubating with rat-anti-mouse Mac-1 [clone M1/70], Ly6G (BD Pharmingen, San Diego, California, USA , 1:50) and rat-anti-mouse Ly-6C [clone NIMP-R14] (Hycult Biotech, Uden, the Netherlands, 1:100), respectively for 1h. Next, slides were incubated with polyclonal biotinylated rabbit anti-rat IgG as secondary antibody (1:200) for 1 hour followed by peroxidase detection using the Avidin Biotin Complex (ABC) kit (Vector Laboratories, Burlingame, USA) for 30 min. The 3-Amino-9-ethylcarbazole (AEC) peroxidase substrate kit (Bio-connect, Huissen, The Netherlands) was used as color substrate to give the red color in presence of peroxidase. Hematoxylin (Klinipath, Duiven, the Netherlands) was used for nuclear counterstaining. Sections were mounted with Faramount aqueous mounting medium (DAKO, Glostrup, Denmark). Washing between all steps was performed using PBS (3x3min). Pictures were taken with a Nikon digital camera DMX1200 and ACT-1 v2.63 software (Nikon Corporation, Tokyo, Japan). The number of positive-stained cells was counted in six pictures (200×) per liver per mouse to determine the level of liver inflammation.

4.5. Flow cytometry

PEC were harvested by peritoneal lavage using ice-cold HBSS + 2% FCS or PBS + 1% BSA. Peripheral blood leukocytes were isolated by incubating blood collected into EDTA tubes with PBS containing 2% Dextran (Sigma-Aldrich) at a 1:1 ratio for 40 min at 37ºC to separate red blood cells. Splenocytes were harvested and meshed through a cell strainer to obtain a single cell suspension and red blood cells were lysed using a commercial lysis buffer (Morphisto). Bone marrow cells were harvested as described above. For flow cytometric analyses, cells were blocked with anti-mouse CD16/32 blocking Ab (anti-mouse CD16/CD32, clone 93, 0.5 µg/0.5 × 106 cells, eBioscience) for 20 min at 4°C and stained for 30 min with following antibodies: PerCP-Cy5.5-labeled anti-CD45R (B220) (clone RA3-6B2, 1:800, eBioscience), FITC-labeled anti-CD23 (clone B3B4, 1:600, BD Biosciences – Pharmingen), APC-labeled anti-IgM (clone

II/41, 1:600, eBioscience), phycoerythrin (PE)-labeled anti-CD43 (clone S7, 1:600, BD Biosciences – Pharmingen), biotin-labeled anti-CD21/CD35 (CR2/CR1) (clone 7E9, 1:200, BioLegend), PE-labeled anti-CD3e (clone 145-2C11, 1:400, eBioscience), FITC-labeled anti-CD4 (clone GK1.5, 1:400, eBioscience), APC-labeled anti-CD8a (clone 53-6.7, 1:400, eBioscience), FITC-labeled anti-kappa (clone 197.1, 1:400, BD Biosciences – Pharmingen), Biotin-labeled anti-lambda (clone RML-42, 1:400, BioLegend), PE-labeled anti-IgD (clone 11-26c (11-26), 1:200, eBioscience), PE-labeled anti-CD93 (clone AA4.1, 1:400, eBioscience), APC-labeled anti-Siglec-G (clone SH2.1, 1:400, eBioscience), APC-labeled anti-CD11b/Mac-1 (clone M1/70, 1:600, eBioscience), FITC-labeled anti CD11b (clone M1/70, 1:600, eBioscience), PE-labeled anti-CD5 (clone 53-7.3, 1:100, eBioscience), FITC-labeled anti-Ly6C (clone HK14, 1:200, BioLegend), PE-labeled anti-Ly6G (clone 1A8, 1:2000, BioLegend), V500-labeled anti-CD45 (clone 30-F11, 1:200, BD Biosciences – Pharmingen), BV570-labeled anti-Ly-6C (clone HK1.4, 1:100, BioLegend), eFluor 780-labeled fixable viability dye (1:1000, eBioscience), AF700-labeled anti-CD11b (clone M1/70, 1:700, eBioscience), Brilliant Violet 570-labelled anti-CD19 (1:100, clone 6D5, BioLegend), PE-labelled anti-GM-CSF (1:100, clone MP1-22E9, BioLegend), PECy7-labelled anti CD93 (1:100, clone AA4.1, eBioscience), biotin-labeled anti-CD5 (Ly-1) (1:200, clone 53-7.3, BD Pharmingen), AF700-labelled anti-MHCII (1:100, clone M5/114.15.2, eBioscience), FITC-labeled anti-IgD (1:800, clone 11-26c, eBioscience), biotin-labeled anti-CD138 (Syndecan 1) (1:200, clone 281-2, BioLegend). All stains were performed in 100 µl of FACS buffer (PBS + 10% FCS) for 30 min at 4°C in darkness, followed by two washing steps. Stained cell populations were analyzed by multiparameter flow cytometry using a BD FACSCalibur (BD Bioscience, Franklin Lakes, New Jersey, USA) or BD FACS Fortessa, respectively. Either 1×10^5 or 1×10^6 cells per sample were stained and acquired. Dead cells and doublets were excluded by forward- and side-scatter and data were analyzed using the FlowJo version 10 data analysis software (Tree Star Inc., Oregon Corporation, Ashland, USA).

4.6. Clinical chemistry and lipid analyses

Total serum cholesterol and triglycerides were measured by enzymatic methods using an automated analyzer AU5400 – Chemistry System (Beckman Coulter, Brea, California, USA). For homogenization of liver lipids, frozen liver tissue (~50 mg) was transferred to a tube containing 1 mm glass-beads with 1 ml SET buffer (250 mmol/L sucrose, 2 mmol/L EDTA, and 10 mmol/L Tris) and spun for 30 s at 5,000 rpm, followed by three freeze-thaw cycles. Liver lipid levels were measured by enzymatic methods with the following commercial kits: The liver

cholesterol content was quantified using the CHOD-PAP assay (Roche Diagnostics, Basel, Switzerland). Liver triglycerides were determined using a serum triglyceride determination kit (Sigma-Aldrich, St. Louis, Missouri, USA). In order to measure non-esterified (or free) fatty acids (NEFA or FFA) in the liver, the NEFA-C kit (ACS-ACOD method; Wako Chemicals, Neuss, Germany) was used. All measurements were done according to the manufacturer's protocols using a Benchmark 550 Micro-plate Reader (Bio-Rad, Hercules, CA). Protein content was measured with the bicinchoninic acid (BCA) method (Pierce, Rockford, IL) according to manufacturer's protocol. Data are expressed as µg lipid per µg protein.

4.7. Gene expression analysis

Total RNA was isolated from ~25 mg of mouse liver tissue and was reversely transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, USA). Quantitative real-time PCR was performed using SensiMix™ SYBR green with ROX dye (Bioline, Randolph, USA) on an ABI7900HT instrument (Applied Biosystems). The relative expression level of target genes was determined with the LinRegPCR software (http://www.hfrc.nl, (Ruijter et al, 2009)), and obtained values were normalized to the housekeeping gene ribosomal protein S12 (Rps12). Values are expressed as the relative expression compared to the control group.

Following primer sequences were used:

Cxcl2-forward: 5'-AGTGAACTGCGCTGTCAATGC-3';
Cxcl2-reverse: 5'-AGGCAAACTTTTTGACCGCC-3';
TNF-α-forward: 5'-CATCTTCTCAAAATTCGAGTGACAA-3';
TNF-α-reverse: 5'-TGGGAGTAGACAAGGTACAACCC-3';
Cxcl1-forward: 5'-TGCACCCAAACCGAAGTCAT-3';
Cxcl1-reverse: 5'-TTGTCAGAAGCCAGCGTTCAC-3';
SAA1-forward: 5'-GGCTGCTGAGAAAATCAGTGATG-3';
SAA1-reverse: 5'-TCAGCAATGGTGTCCTCATGTC-3';
VCAM-forward: 5'-GTGTTGAGCTCTGTGGGTTTTG-3';
VCAM-reverse: 5'-TTAATTACTGGATCTTCAGGGAATGAG-3';
ICAM-forward: 5'-CTACCATCACCGTGTATTCGTTTC-3';
ICAM-reverse: 5'-CGGTGCTCCACCATCCA-3';
IL-18-forward: 5'-ACAACTTTGGCCGACTTCAC-3';
IL-18-reverse: 5'-GGGTTCACTGGCACTTTGAT-3';
CCL5-forward: 5'-GGAGTATTTCTACACCAGCAGCAA-3';

CCL5-reverse: 5'-GCGGTTCCTTCGAGTGACA-3';
S12-forward: 5'-GGAAGGCATAGCTGCTGGAGGTGT-3';
S12-reverse: 5'-CCTTCGATGACATCCTTGGCCTGAG-3'.

4.8. Validation of successful BMT

At time of sacrifice, bone marrow cells from all mice were collected and genomic DNA was extracted by incubating cells in 40 µl of lysis buffer (1 M Tris/HCl, pH 8.0, 500 mM EDTA, 10% SDS, 2 M NaCl, 0.4 mg/ml Proteinase K) at 55°C overnight with gentle mixing, followed by 10 min incubation at 99°C to inactivate Proteinase K. The purified genomic DNA was diluted 1:10 in H_2O and amplified by PCR for the Ldlr and Siglec-G gene to identify successful bone marrow reconstitution with donor bone marrow.
In order to assess selective Siglec-G deficiency on B cells, B cells and non B cells of splenocytes from recipient mice were separated using anti-CD45R (B220) MicroBeads (Milteny Biotec) according to the manufacturer's protocol. Collected cell fractions were lysed in RLT Plus buffer (Qiagen) and stored at -20°C. Total RNA and genomic DNA were simultaneously purified with the AllPrep DNA/RNA Micro Kit (Qiagen) according to the manufacturer's protocol and subsequently used for PCR to amplify the genes of interest, as described below:

Siglec-G-forward: 5'-CCGCTCGAGATGTTGTCCCGCGGGTGGTTTCA-3';
Siglec-G-reverse: 5'-CCGGAATTCCTTCCTCTTGAGAGAACCTTTGTTC-3';
Ldlr-forward: 5'-ACCCCAAGACGTGCTCCCAGGATG-3';
Ldlr-reverse: 5'-CGCAGTGCTCCTCATCTGACTTGT-3';
Neo cassette: 5'-AGGTGAGATGACAGGAGA-3'.

4.9. Plasma antibody and protein analyses

Total IgM levels were measured by a chemiluminescent-based sandwich ELISA using an anti-mouse IgM capture antibody (M8644, Sigma-Aldrich) coated on 96-well microtitration plates (Thermo, MicrofluorII roundbottom) in 40 µl PBS at 2 µg/ml, overnight at 4°C. Plasma was diluted 1:30,000 in PBS and a mouse IgM isotype control (401602, BioLegend) was used for the standard curve in 2-fold dilutions, starting at 50 ng/ml (incubated overnight at 4°C or 2 hours room temperature). For detection, an AP-conjugated goat anti-mouse IgM (1:20,000 in TBS-BSA, A9688, Sigma-Aldrich; incubated 2 hrs at room temperature) and Lumiphos Plus (P-701, 30% solution in water, Lumigen, South Field, Michigan, USA; incubated 1 hr at room

temperature) were used. All washing steps were performed on a microplate washer ELx405 (BioTek) with PBS or PBS-EDTA. The results were expressed as relative light units (RLU) per 100ms. Chemiluminescence was detected using a luminescence reader (BioTek Synergy 2, BioTek, Winooski, Vermont, USA).

Total IgG levels were measured in plasma diluted 1:70,000 in Assay Buffer A using a commercially available ELISA (Mouse IgG total ELISA Ready-SET-Go!, eBioscience). All steps were performed according to manufacturer's instructions.

The antigens were prepared as described previously (Chou et al, 2009; Palinski et al, 1990). Copper-oxidized LDL (CuOx-LDL) and malondialdehyde-modified LDL (MDA-LDL) was prepared from human LDL isolated from pooled plasma of healthy donors. MAA-BSA was prepared by modification of bovine serum albumin (BSA) with MDA and acetaldehyde as described (Xu et al, 1997). BSA and KLH were purchased from Pierce Biotechnology. AB1-2, an anti–T15 idiotype Ab, was provided by J. Kearney (University of Alabama at Birmingham, Birmingham, Alabama, USA).
Antigen-specific antibody titers were measured by chemiluminescent ELISA as previously described (Binder et al, 2003). In brief, antigens were coated on 96-well white, round bottom microtitration plate (Thermo, MicrofluorII roundbottom) at a concentration of 5 µg/ml in PBS containing 0.27 mM EDTA and incubated for 1 hour at 37°C or overnight at 4°C. After washing and blocking steps (in TBS pH 7.4, containing 1% BSA and 0.27 mM EDTA), the plate was incubated with plasma samples in respective dilutions (anti–T15 idiotypic antibody AB1-2 IgM: 1:100, PC-BSA IgM: 1:100, CuOx-LDL IgM: 1:500, MDA-LDL IgM: 1:500, α-1,3-dextran 1:100) in TBS-BSA (TBS pH 7.4, containing 1% BSA and 0.27mM EDTA) for 2 hrs at room temperature or overnight at 4°C. Mouse anti-goat-IgM conjugated to alkaline phosphatase (AP) (SigmaAldrich, 1:30,000 in TBS-BSA) was used for detection. AP-conjugated secondary reagents were detected by using Lumiphos (Lumigen, 33% solution in water) and a Dynex Luminometer (Dynex Technologies). Washing steps were performed on a microplate washer ELx405 (BioTek) with PBS or PBS-EDTA. The results were expressed as relative light units (RLU) per 100ms.
OxLDL-specific IgM per total IgM ratios were calculated based on the measurements of specific IgM at non-saturating dilutions (expressed as RLU/100ms) and total IgM quantities of each individual sample. Data are expressed as arbitrary units of these ratios.

SAA levels were measured in plasma diluted 1:300 in diluent buffer using a commercially available ELISA for mouse SAA, in accordance with the manufacturer's instructions (Tridelta Development Ltd.).

4.10. Measurement of chemokines/cytokines in plasma and peritoneal lavage fluid

A panel of chemokines and cytokines (IL-6, TNF-α, CCXL1, CXCL2, CCL2 and IL-18) was measured in mouse plasma (1:2 dilution) with ProcartaPlex® Multiplex Immunoassays (eBioscience) according to the manufacturer's protocol. Analysis was performed with the xMAP® technology by Luminex (Austin, TX, USA). The chemokines CXCL1 and CXCL2 were quantified in peritoneal lavage fluids (undiluted) by using the commercially available mouse CXCL1/KC DuoSet ELISA and Mouse CXCL2/MIP-2 DuoSet ELISA, respectively according to the manufacturer's protocol (R&D systems, Minneapolis, MN, USA).

4.11. Murine sterile peritonitis

Siglec-G$^{-/-}$ mice and their wild type littermate controls (8–12 weeks of age) were injected *i.p.* with sterile OxLDL (tested for endotoxin levels by chromogenic Limulus amoebocyte assay; < 0.05 ng LPS/mg protein) suspended in 600 µl of sterile PBS+1% BSA at a concentration of 25 µg per gram body weight or a comparable volume of sterile thioglycollate (Lipid MAPS) (25 µl/g body weight). At selected time points, mice were sacrificed, and peritoneal exudates were collected by lavaging the peritoneum with 6 ml of sterile PBS+1% BSA. PEC were pelleted for 10 min at 300 g, and cells were subsequently processed for flow cytometry, as described above. Cell-free lavage fluid was further used for ELISA measurements.

4.12. Statistical analysis

Data were analyzed using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA). Normal distribution of data was assessed, and statistical analyses were performed by unpaired Student *t*-test for all results of *in vivo* studies to determine statistical significance between the groups. Data points, which were above or below more than 2xSTDEV of the mean, were excluded as statistical outliers. Of note, exclusion of these values did not change the significance of the results. Data are presented as the mean ± standard error of mean and considered significant at $P < .05$ ($^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$, respectively).

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Sialic acid-binding immunoglobulin-like lectin G promotes atherosclerosis and liver inflammation by suppressing the protective functions of B-1 cells.

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